Factores inmunológicos asociados a la aparición de eventos no SIDA y al control espontáneo de la infección por el VIH

Tesis Doctoral

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UNIVERSIDAD DE SEVILLA

Facultad de Medicina

Doctorado en Biología Molecular, Biomedicina e Investigación Clínica

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Certifican:

Que el trabajo titulado "*Factores inmunológicos asociados a la aparición de eventos no SIDA y al control espontáneo de la infección por el VIH*" presentado por LAURA TARANCÓN DÍEZ, ha sido realizado bajo nuestra dirección y asesoramiento en el Laboratorio de Inmunovirología del Instituto de Biomedicina de Sevilla (IBiS) y autorizamos la presentación y la defensa de esta Tesis Doctoral, para que sea evaluada por el tribunal correspondiente.

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Fdo. Dr. Manuel Leal Noval

Fdo. Dr. Ezequiel Ruiz-Mateos Carmona

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CONTENIDO

INTRODUCCIÓN

La mayoría de los pacientes infectados por VIH (90%) se encuentran actualmente bajo tratamiento antirretroviral combinado (TARc) supresor. Desde 1996, gracias a la introducción y eficacia de estos tratamientos, las causas de mortalidad debidas a SIDA se han reducido considerablemente (1) y actualmente, la mayoría de las causas de muerte en pacientes infectados por VIH bajo TARc supresor se deben a un conjunto de patologías denominadas eventos no definitorios de SIDA (ENOS) (Figura 1). El principal ENOS es la enfermedad hepática terminal la cual, sin embargo, ha perdido relevancia en los últimos años gracias a la aparición de los nuevos tratamientos antivirales directos frente al virus de la hepatitis C (VHC). De gran importancia son la enfermedad cardiovascular, el cáncer y las infecciones bacterianas, enfermedades que también se observan en la población general anciana no infectada. De hecho, se considera que los sujetos infectados por VIH podrían estar sufriendo una senescencia prematura que además explicaría la menor esperanza de vida que se observa en estos sujetos en comparación con la población general (2, 3). Los mecanismos inmunopatogénicos involucrados en el desarrollo de ENOS cardiovasculares no son bien conocidos, aunque en base a estudios de fenotipo celular y biomarcadores (4, 5) se ha propuesto que en estos pacientes existe un estado inflamatorio crónico de bajo grado debido a una activación sostenida del sistema inmunitario innato, que el TARc supresor no restaura a la normalidad. Intentando esclarecer el origen de esta activación crónica, altos niveles de biomarcadores solubles de inflamación se han asociado a la aparición de ENOS (6) y en esta línea de trabajo nuestro grupo observó cómo parámetros asociados con el monocito, componente del sistema inmunitario innato, no se normalizaban tras un TARc supresor. Estos parámetros fueron el TNF-α, cuyos altos niveles fueron similares a sujetos no infectados con una mediana de edad de 80 años (7), el CD163 (8) y el CD14 soluble (9). Tras una caracterización más exhaustiva de los monocitos descubrimos

una profunda afectación de la función de este tipo celular en pacientes infectados por VIH en comparación con sujetos ancianos debido a altos niveles de activación y de citoquinas proinflamatorias producidas *ex vivo* e *in vitro* en respuesta a estímulos específicos para *Toll like receptor* (TLR)-2, -7 así como el TLR4, receptor del lipopolisacárido bacteriano (LPS) (10).

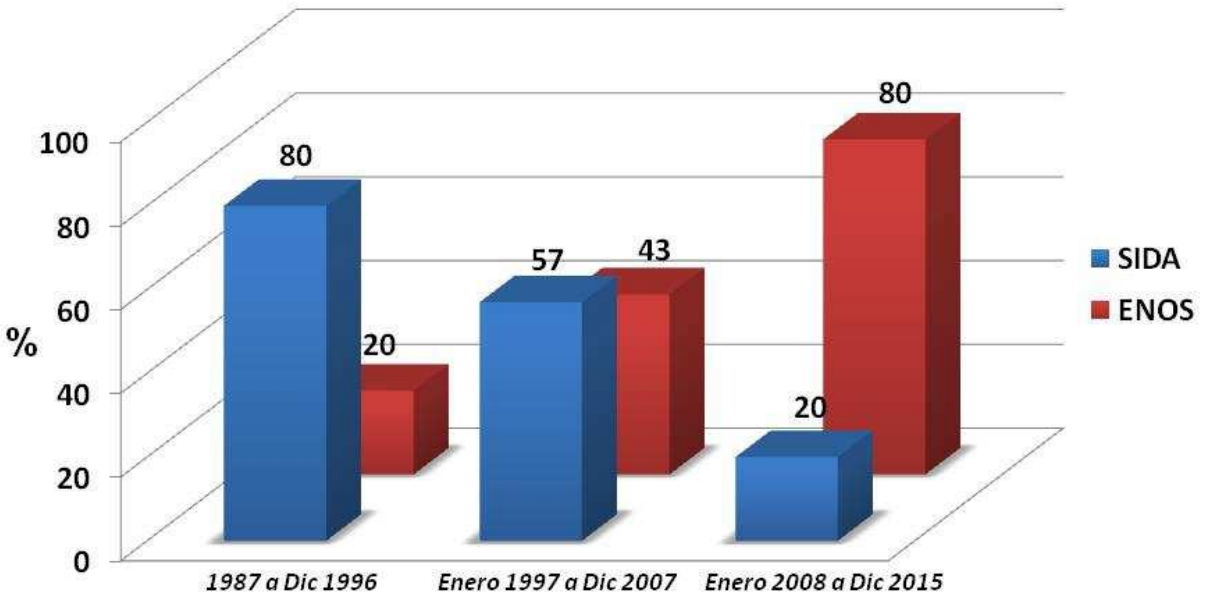

Figura 1: Causas de muerte a lo largo de la historia del tratamiento antirretroviral. Datos obtenidos de 222 pacientes que acudieron al Hospital Universitario Virgen del Rocío.

Otro importante componente del sistema inmunitario innato que, junto con el monocito, juega un papel fundamental en la inmunopatogenia de la infección por VIH es la célula plasmocitoide dendrítica (pDC) (11, 12). Estas células, a pesar de representar únicamente el 1% de las células mononucleares de sangre periférica (PBMCs), responden a las infecciones víricas produciendo grandes cantidades de interferón alpha (IFN-α) tras la estimulación de receptores endosómicos TLR7 y TLR9 (11, 13). Además,

las pDCs se consideran un enlace entre la inmunidad innata y adaptativa, induciendo y manteniendo una respuesta efectiva en células T antígeno-específica (14, 15). La activación permanente de la pDC en el escenario de la infección por el VIH que resulta en una producción aberrante de IFN-α podría explicar una hiperinflamación crónica de bajo grado responsable del daño celular y tisular y por ende asociada al incremento en la prevalencia de ENOS en estos pacientes.

Actualmente las enfermedades cardiovasculares aterogénicas (ECA) son las principales causas de morbimortalidad en el paciente infectado por VIH (16). La interacción entre los factores de riesgo cardiovascular tradicionales (17) así como el efecto de algunos tipos de terapias antirretrovirales (18), se han asociado tradicionalmente con mayor prevalencia de eventos cardiovasculares; sin embargo, estudios recientes destacan la importancia de la activación monocitaria y de linfocitos T (5, 19) como otro mecanismo involucrado en estos procesos. La activación de linfocitos T se ha estudiado en relación con enfermedad cardiovascular en población VIH (19–21), sin embargo estos trabajos no han analizado el fenotipo de los linfocitos T y su asociación con componentes del sistema innato. Atendiendo a esta rama del sistema inmunitario, la implicación de pDCs y monocitos en estos eventos se ha observado en estudios *in vitro*, modelos animales y en población general (22, 23). En relación con los monocitos, mutaciones en el receptor TLR4 también se han asociado con el desarrollo de ECAs en población general (24, 25). Por todo ello, la implicación y asociación de ambas ramas del sistema inmunitario, adaptativo e innato, en la aparición de ECAs en el escenario de la infección por el VIH no era bien conocido. Esta laguna de conocimiento ha sido abordada en los siguientes trabajos: “CCR5+ CD8 T-cell levels and monocyte activation precede the onset of acute coronary syndrome in HIV-infected patients on

antiretroviral therapy" (*Thrombosis and haemostasis*, 2017) y "Role of TLR4 Asp299Gly polymorphism in the development of cardiovascular diseases in HIV-infected patients" (*AIDS*, 2018**)**.

Observaciones sobre el papel de las infecciones agudas en el riesgo de ECAs en población general no infectada (26) han sugerido la posible implicación de coinfecciones en el escenario del VIH como otro posible factor implicado en la aparición de las ECAs. El papel de las infecciones crónicas como la coinfección por el VHC en el desarrollo de ECAs también se ha estudiado como otro posible factor implicado aunque con resultados contradictorios (27–29). En esta línea, la reactivación por herpes zoster, la pneumonia bacteriana y la acumulación de infecciones per sé, podrían también estar asociadas con la aparición de estas patologías (30–32), aunque hasta donde sabemos no se ha estudiado en el contexto de la infección por VIH. El estudio de la potencial asociación de la coinfección por VHC y el acumulo de infecciones ha sido abordado en el trabajo "Hepatitis C virus and cumulative infections are associated with atherogenic cardiovascular events in HIV-infected subjects" (*Antiviral Research*, 2019).

Una de las principales moléculas que aumenta su expresión en el proceso de activación de monocitos y linfocitos T es el receptor CCR5 (33). Curiosamente, la deleción Δ32 en el gen CCR5, (CCR5Δ32) además de ser un factor resistente a la infección por VIH en homozygosis, también se ha asociado a un menor riesgo de enfermedad coronaria en población general (34) y en el escenario del VIH, trabajos seminales demostraron que pacientes heterocigotos progresaban más lentamente a SIDA y experimentaban menor muerte cruda que individuos con los alelos silvestres para la mutación (35–37). Estos trabajos se llevaron a cabo cuando aún no se disponía de un TARc óptimo (mediados años 90), sin embargo, la influencia de la deleción CCR5Δ32 en la progresión clínica y mortalidad en la nueva era de

Tratamientos Antirretrovirales de Gran Actividad (TARGA) a largo plazo no era bien conocido. Este objetivo se abordó en el manuscrito "Association of heterozygous CCR5Δ32 deletion with survival in HIV- infection: A cohort study" (*Antiviral Research*, 2018).

Los trabajos previamente descritos corresponden con lo que ocurre en la mayoría de los pacientes infectados por VIH, sin embargo, hay una pequeña fracción de pacientes (1%) que tienen la extraordinaria capacidad de mantener espontáneamente niveles bajos ("controladores virémicos") o indetectables de carga viral plasmática ("controladores de élite", CE) durante largos periodos de tiempo en ausencia de TARc; conjuntamente denominados "Controladores del VIH" (38). A pesar del continuo debate en la comunidad científica acerca de si estos pacientes necesitan TARc los CE generalmente no progresan, mantienen altos niveles de células T-CD4+ y poseen una inmunidad preservada (39) por lo que han sido propuestos como un modelo de cura funcional (40, 41).

Nosotros, entre otros grupos, hemos participado en esclarecer los principales mecanismos implicados en este fenómeno, por un lado asociados a la genética del huésped (42–44) y por otro a la inmunidad adaptativa e innata (45–47).

Una de las principales limitaciones de los trabajos realizados hasta el momento en CE es que se han realizado únicamente en estudios transversales de comparación de grupos: no pudiéndose establecer la causalidad de estas variables en el fenómeno controlador. Cada vez más evidencias apuntan a un fenotipo heterogéneo en estos pacientes que incluye progresión virológica, inmunológica, así como el desarrollo de ENOS (48–50). En el escenario de la progresión virológica, aproximadamente un 24% de

los CE experimentan pérdida de control virológico espontáneo a lo largo del seguimiento (49), pero los mecanismos involucrados en la pérdida del control no son bien conocidos. Conocer los mecanismos inmunovirológicos responsables de la pérdida de control virológico en un seguimiento longitudinal de estos pacientes podría ayudar a restablecer una correcta definición de CE persistente con el fin de lograr una posible remisión virológica a largo plazo o cura funcional en los pacientes infectados por VIH, así como anticipar una intervención terapéutica en aquellos pacientes que van a perder el control. Este objetivo se ha realizado gracias al Grupo de Estudio de Pacientes Controladores de la RIS (EC-RIS) y a la Cohorte Española de Pacientes VIH Controladores (ECRIS-DB) coordinada por nuestro grupo y que incluye en la última actualización 807 pacientes controladores del VIH. Estos objetivos se han abordado en los trabajos "Factors leading to the loss of natural elite control of HIV-1 infection" (*Journal of Virology*, 2017), "Proteomic profile associated with loss of spontaneous HIV-1 elite control" (*The Journal of Infectious Diseases*, 2019) and "Immunometabolism is a key factor for the persistent spontaneous elite control of HIV-1 infection" (*EBioMedicine*, 2019).

OBJETIVOS

Hipótesis 1: El desarrollo de ENOS cardiovasculares en la población infectada por VIH puede estar asociado con determinadas alteraciones fenotípicas y funcionales de componentes del sistema inmunitario innato y adaptativo así como con otras coinfecciones.

Objetivo 1: Investigar la relación entre el fenotipo de células del sistema inmunitario innato (monocitos y pDCs) y adaptativo (linfocitos T-CD4 y CD8), el polimorfismo Asp299Gly en el receptor TLR4 y otras coinfecciones (VHC, reactivación de Herpes Zoster e infecciones bacterianas) con la aparición de enfermedades cardiovasculares aterogénicas en pacientes infectados por VIH. Este objetivo fue analizado en los trabajos **"CCR5+ CD8 T-cell levels and monocyte activation precede the onset of acute coronary syndrome in HIV-infected patients on antiretroviral therapy"** (*Thrombosis and Haemostasis*, 2017), **"Role of TLR4 Asp299Gly polymorphism in the development of cardiovascular diseases in HIV-infected patients"** (*AIDS*, 2018) y **"Hepatitis C virus and cumulative infections are associated with atherogenic cardiovascular events in HIV-infected subjects"** (*Antiviral Research*, 2019).

Hipótesis 2: Pacientes con la deleción CCR5Δ32 en el receptor CCR5 presentan un curso clínico más favorable y menor mortalidad que pacientes infectados por VIH que portan el genotipo silvestre para esta deleción.

Objetivo 2: Analizar la influencia de la deleción CCR5Δ32 en la progresión clínica y mortalidad en una gran cohorte de pacientes infectados por VIH incluyendo la era TARGA. Este objetivo se abordó en el manuscrito "**Association of heterozygous CCR5Δ32 deletion with survival in HIV- infection: A**

cohort study" (*Antiviral Research*, 2018).

Hipótesis 3: Distintos factores inmunológicos y virológicos son responsables de la pérdida del control virológico espontáneo en los pacientes VIH controladores de élite.

Objetivo 3: Analizar longitudinalmente mecanismos inmunológicos y virológicos implicados en la pérdida de control virológico en pacientes controladores de élite. Este objetivo se estudió en los trabajos: "**Factors leading to the loss of natural elite control of HIV-1 infection**" (*Journal of Virology*, 2017), "**Proteomic profile associated with loss of spontaneous HIV-1 elite control**" (*The Journal of Infectious Diseases,* 2019) and "**Immunometabolism is a key factor for the persistent spontaneous elite control of HIV-1 infection**" (*EBioMedicine*, 2019).

RESUMEN GLOBAL DE RESULTADOS

CCR5+ CD8 T-cell levels and monocyte activation precede the onset of acute coronary syndrome in HIV-infected patients on antiretroviral therapy

Tarancon-Diez L *et al*

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CCR5+ CD8 T-cell levels and monocyte activation precede the onset of acute coronary syndrome in HIV-infected patients on antiretroviral therapy

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Summary

Acute coronary syndrome (ACS) is nowadays one of the leading causes of morbid-mortality in HIV-infected population, but innate and adaptive immune mechanisms preceding this event are unknown. In this work we comprehensively and longitudinally observed, by multiparametric flow cytometry and following a case-control design, increased CCR5$^+$CD8$^+$ T-cells levels and monocytes expressing activation and adhesion markers in HIV-infected patients who are going to suffer ACS. In addition, we found direct associations between activated CD8$^+$ T-cells and myeloid cells that were only statistically significant in the group of patients with ACS and in the follow up time point just before the ACS. Our data highlight the important role of CCR5 in the onset of ACS and suggest this receptor as a marker of cardiovascular risk and potential therapeutic target to prevent the development of such non-AIDS-related event in HIV-infected patients.

Keywords

HIV, CCR5, monocyte, vascular event, immune activation

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Introduction

The introduction of combined antiretroviral therapy (cART) dramatically reduced AIDS-related morbidity and mortality. However, non-AIDS-related events, such as those attributable to atherosclerotic cardiovascular diseases (CVDs), have become increasingly relevant, increasing approximately two-fold compared to a non-HIV-infected population and becoming one of the leading causes of morbidity and mortality in HIV-infected patients (1). In this context, the higher prevalence of acute coronary syndrome (ACS) has been related to the interplay between cART (2) and traditional CVD risk factors (3); however, more recent studies have focused on the importance of inflammation and immune activation (4) as another main source of these diseases.

In a non-HIV-infected population, although there are no detailed studies collectively analysing the different phenotypes of adaptive and innate immune system components, separately, CVDs have been found to be associated with both arms of immunity in cross-sectional studies (5). The immunomodulation of distinct monocyte subsets (6) and CD4 and CD8 T-cells (7) have recently been discussed as new therapeutic targets in atherosclerotic disease. Regarding HIV-infection, the contribution of

myeloid (8) and T-cells (9) to the progression of CVDs have also been studied in cross-sectional studies using mainly carotid intima-medial thickness (IMT) as surrogate end points, instead of well recorded clinical end points, showing controversial results (10, 11).

As far as we know, there are no longitudinal analyses regarding the association and contribution of both innate and adaptive immune systems using well-characterised clinical end points such as ACS in an HIV-infected population. Therefore, the aim of the present study was to longitudinally and comprehensively investigate the relationship between innate (monocytes, dendritic cell subsets, and neutrophils) and adaptive immune cells (CD4 and CD8 T-cells) and the occurrence of ACS in HIV-infected patients on suppressive cART.

Material and methods

Study subjects

Asymptomatic HIV-infected patients visiting the Virgen del Rocío University Hospital in Seville between 2005 and 2013 were included in this case control study. Patients were on suppressive cART for at least six months prior to the first episode of ACS (defined as acute myocardial infarction or unstable angina), had no previous recorded CDC-C or non-AIDS-related illnesses and had available peripheral blood mononuclear cell (PBMC) cryopreserved samples. Clinical data, epidemiological data and cardiovascular risk factors (including smoking, diabetes mellitus and high blood pressure prevalence) were recorded. In addition, the 10-year Framingham risk score was calculated (12). These patients were referred to as the Case group (n=16) and were matched by age, sex, CD4 T-cell count, nadir CD4 T-cell count, time since HIV diagnosis and RNA+ for hepatitis C virus (HCV) with a Control group (n=16) that fulfilled the criteria defined above but had not suffered ACS. Frozen isolated PBMCs from the Case group were retrospectively selected 48, 24 weeks and immediately before (defined as the "pre" time-point: 4, [1–7] weeks, median and IQR) the ACS. PBMCs from the Control group were selected from one time-point. All samples were obtained from the HIV Biobank of the Spanish AIDS Research Network. Laboratory evaluations were performed at the Laboratory of Immunovirology at the Institute of Biomedicine of Seville (IBiS) of Virgen del Rocío University Hospital in Seville (Spain). Institutional and Ethical Review Board approvals were obtained and written informed consent was obtained from all study patients.

Laboratory methods

Monocyte, neutrophil, CD4 T-cell and CD8 T-cell absolute counts were determined in fresh whole blood using an Epic XL-MCL flow cytometer (Beckman-Coulter, Brea, CA, USA) according to the manufacturer's instructions. Plasma HIV-1 RNA concentration was measured using quantitative polymerase chain reaction (COBAS Ampliprep/COBAS Taqman HIV-1 test, Roche Molecular Systems, Basel, Switzerland) according to the manufacturer's protocol. The detection limit for this assay was 20 HIV RNA copies/ml. Hepatitis C virus (HCV) RNA was determined using an available PCR procedure kit (COBAS Amplicor, Roche Diagnosis, Barcelona, Spain) with a detection limit of 10 IU/ml. Cytomegalovirus (CMV) exposition was measured by positive plasmatic IgG antibodies (Elecsys CMV IgG, Roche Molecular Systems).

Immunophenotyping of monocytes, dendritic cells and T lymphocytes

Immunophenotyping of dendritic cells, monocytes and T lymphocytes using multiparametric flow cytometry was performed following this hierarchy order according to sample availability (in future analysis, the number of patients will vary due to that limitation). Briefly, PBMCs were thawed, washed and stained with surface and intracellular marker antibodies (see the Supplementary material). Isotype controls were included in each experiment. Multiparametric flow citometry were done using a LSR Fortessa Cell Analyser (BD Biosciences, Franklin Lakes, NJ, USA). A minimum of 1,500,000 total events were recorded for each panel.

Assay of inflammatory and coagulation biomarkers and soluble CCR5 ligands

High-sensitivity C reactive protein (hsCRP) and β2-microglobulin were determined with an immunoturbidimetric assay using COBAS 701 (Roche Diagnostics, GmbH, Mannheim, Germany). D-dimer levels were determined using an automated latex enhanced immunoassay (HemosIL, D-Dimer HS 500, Instrumentation Laboratory, Bedford, MA, USA) in plasma samples stored at -20ºC. The measurement of CCR5 ligands, MIP-1α (CCL3), MIP-1β (CCL4) and RANTES (CCL5) was assayed in serum samples stored at -20ºC using an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, San Diego, CA, USA).

IFN-α production by PBMCs

Frozen isolated PBMCs (0.5×10^6 cells) were cultured in a 48-well plate overnight with R10 media (RPMI 1640 supplemented with 10% heat-inactivated calf serum, 100 µl/ml streptomycin sulfate and 1.7 Mm sodium glutamine) and stimulated with 1 µM CpG ODN2216 (InvivoGen, San Diego, CA, USA), a Toll-like receptor 9 (TLR9) agonist. The amount of IFN-α in the supernatants was quantified using an IFN-α multisubtype ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ, USA) according to the manufacturer's instructions.

Statistical analysis

Continuous variables were expressed as the medians and interquartile ranges (IQR). Categorical variables were expressed as numbers and percentages. Correlations between variables were assessed using Spearman's rank test. Differences between categorical values were determined using the chi-square test. Differences between unpaired groups were determined using the Mann Whitney

U-test and differences between paired samples were determined using Wilcoxon signed rank and Friedman tests. P-values < 0.05 were considered statistically significant. The Statistical Package for the Social Sciences software (SPSS 20.0; SPSS, Chicago, IL, USA) was used for the statistical analysis. Graphs were generated using GraphPad Prism, version 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Characteristics of the studied subjects

Clinical and demographic characteristics of the Case group (n=16) from the closest time-point previous to the ACS, the *pre* time-point (4 [1–7] weeks, before the event) and the Control group (n=16) are shown in ►Table 1. We observed a trend towards higher levels of the traditional risk factors: triglycerides and Framingham score (p=0.068 and p=0.091, respectively) in the Case group. Additionally, no differences were found in CD8 T-cell counts. Regarding inflammatory biomarkers, we observed a borderline statistical significance towards higher hsCRP levels in the Case group (p=0.073). In contrast, a borderline statistical significance towards lower levels of β2-microglobulin was also found in this group (p=0.051). All studied patients were anti-CMV$^+$. Clinical data from subjects in Control group were recorded during a median of 38 [34–40] months of follow-up after the inclusion in the study and none of the subjects suffered cardiovascular diseases during this observation period.

High CCR5$^+$ CD8 T cell levels precede the occurrence of ACS

We analysed CD4 and CD8 T-cell immunophenotypes (for gating strategy see Suppl. Figure 1A, available online at www.thrombosis-online.com). CCR5$^+$ CD8 T-cell levels were increased at the *pre* time-point in the Case group compared with the control group, including total CD8 T-cells, naive (CD45RA$^+$CD27$^+$), effector (CD45RA$^-$CD27$^-$) and terminally differentiated (CD45RA$^+$CD27$^-$) CD8 T-cells (p<0.01, p=0.03, p=0.03 and p=0.046, respectively) (►Figure 1A-D). Unexpectedly, we did not find differences in soluble CCR5 ligand levels between the two groups (Suppl. Table 1, available online at www.thrombosis-online.com). However, there was a trend to RANTES levels directly associated with CCR5$^+$ CD8 T-cell levels (r=0.45; p=0.05). Additionally, at the *pre* time-point, the Case group had a lower percentage of central memory CD8 T-cells (CD45RA$^-$CD27$^+$; p=0.03) and a higher percentage of terminally differentiated CD8 T-cells (p=0.04). CD38$^+$ central memory CD4 T cell levels were lower compared with Controls (p=0.013). A similar results were observed in the other CD4 T cell subsets (Suppl. Table 2, available online at www.thrombosis-online.com). We did not find statistically significant differences in most of the studied parameters.

Table 1: Characteristics of the study groups.

Characteristics	Case (n=16)	Control (n=16)	P-value
Sex (men)	13 (81)	13 (81)	1
Age (years)	49 [45–52]	48 [43–51]	0.6
Transmission route (sexual)	9 (56)	8 (50)	0.5
CD4 T (cells/mm^3)	587 [431–627]	473 [377–569]	0.2
CD8 T (cells/mm^3)	973 [419–1160]	570 [424–1018]	0.3
Ratio CD4:CD8 (cells/mm^3)	0.5 [0.4–1.4]	0.8 [0.5–1]	0.5
Nadir CD4 T (cells/mm^3)	134 [53–206]	133 [101–168]	0.8
Time since HIV diagnosis (years)	14 [9–19]	11 [7–21]	0.8
HCV RNA detected	8 (50)	8 (50)	1
Anti-CMV IgG detected	16 (100)	16 (100)	1
Total cholesterol (mg/dl)	197 [195–223]	180 [165–205]	0.2
LDL cholesterol (mg/dl)	150 [132–192]	125 [102–146]	0.1
HDL cholesterol (mg/dl)	51 [43–69]	45 [44–65]	0.7
LDL/HDL	2.9 [1.8–4.1]	2.8 [2.3–3.7]	1
Triglycerides (mg/dl)	195 [121–325]	126 [79–206]	0.068
Smoking	9 (56)	5 (31)	0.2
Framingham	15 [5–18]	6 [2–9]	0.091
High blood pressure	2 (13)	2 (13)	1
Diabetes mellitus	4 (25)	1 (6)	0.2
hsCRP (mg/l)	4.5 [1.5–9]	1.1 [0.7–3.2]	0.075
β2-microglobulin (μg/ml)	1.65 [1.5–9]	2.1 [1.7–2.5]	0.051
D-dimer (μg/l)	328 [98–380]	234 [126–383]	0.747

The Case group values are taken from the closest time prior to the ACS "Pre" (4, [1–7] weeks, median and IQR). Continuous variables are expressed as the medians and interquartile ranges (IQR). Categorical variables are expressed as numbers and percentages. Mann–Whitney U-test and Chi-square tests were used.

High monocyte activation levels in patients with ACS during the 48 weeks before the event

The total monocyte count was higher in the Case group at 48 weeks, 24 weeks and *pre* time-point (►Figure 2A) compared with the Control group (p=0.03, p=0.01 and p=0.01, respectively). We used flow cytometry to perform an exhaustive study of monocyte subsets (for gating strategy see Suppl. Figure 1B, available online at www.thrombosis-online.com): the classical monocytes (CD14^{++}CD16$^-$), the intermediate monocytes (CD14^{++}CD16$^+$) and the nonclassical or *patrolling* monocytes (CD14^{dim}CD16$^+$). The full data are shown in Suppl. Table 3 (available online at www.thrombosis-online.com). The most outstanding finding was that higher levels of classical monocytes expressing the activation and cell adhesion markers CCR5, CD11b, CCR2 and CD49d were found not only at the *pre* time-point (p=0.03, p=0.04, p=0.03 and p=0.03, re-

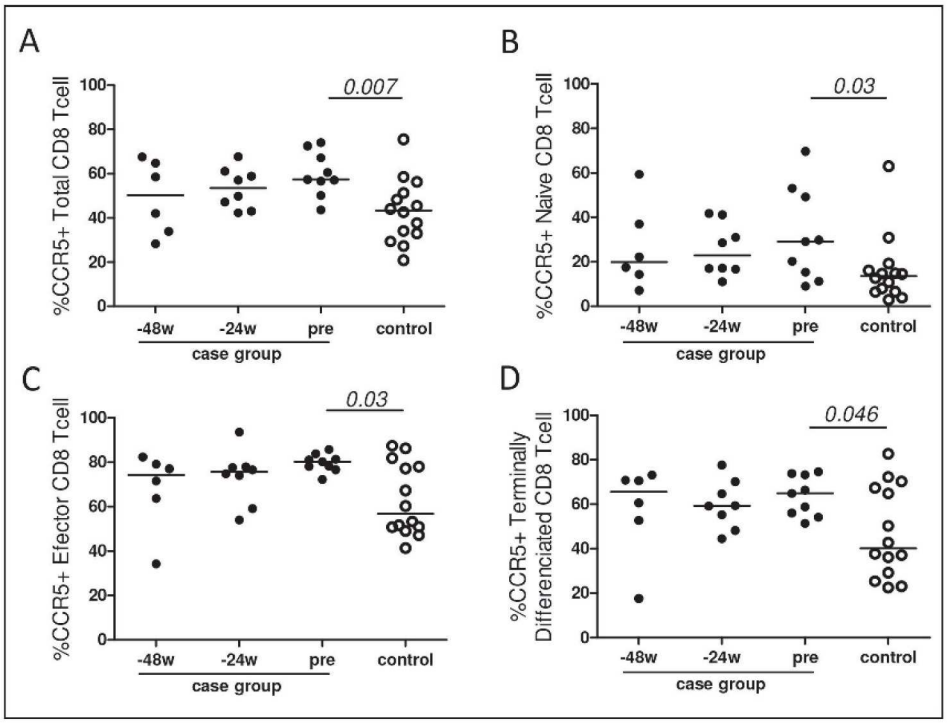

Figure 1: Percentage of total and CCR5⁺ CD8 T-cell subsets. Total (A), naive (B), effector memory (C) and terminally differenciated CD8 T-cells (D). The Mann Whitney U-test was used to compare groups. The Friedman test was not applied due to the small number of paired samples. Only significant differences are shown.

spectively, for each marker) but also 48 weeks before the event compared with the Control group (▶Figure 2B-E). Similar results were found for CD40 (a costimulatory protein) expression, as measured by the median fluorescence intensity (MFI), on intermediate monocytes and CD11b (a leukocyte adhesion and migration mediator) expression on *patrolling* monocytes (p=0.02 and p=0.01, respectively) (▶Figure 2F-G). Regarding surface and intracellular markers in DCs (for gating strategy see Suppl. Figure 1C, available online at www.thrombosis-online.com), no statistically significant differences were found for any of those variables (Suppl. Table 4, available online at www.thrombosis-online.com).

High levels of activated monocytes and DCs are associated with increased CCR5 expression on CD8 T cells of patients with ACS

We next analysed the relationship between CCR5+ CD8 T-cell levels and different innate immune system markers. There was no association between CCR5+ CD8 T-cell levels with myeloid markers in either the Case group at 48 and 24 weeks or in the Control group (▶Figure 3A-C). Interestingly, a separate analysis of the Case group at the closest time-point before the ACS (the *pre* time-point) revealed a strong association between the CCR5⁺ CD8 T-cell levels with the absolute number of classical monocytes expressing the endothelial adhesion marker tissue factor (TF) (r=0.79; p=0.01) (▶Figure 3A), the number of *patrolling* monocytes expressing the homing receptor CCR2 (r=0.85; p<0.01) (▶Figure 3B) and the percentage of myeloid dendritic cells (mDCs) expressing the gut-homing molecule integrin-β7 (r=0.73; p=0.03) (▶Figure 3C).

In addition, CCR5+ CD8 T-cell levels from the Case group together with the Control group correlated with the total number of monocytes and the number of classical monocytes expressing CCR2 (r=0.45; p=0.02 and r=0.41; p=0.04, respectively) (Suppl. Figure 2A-B, available online at www.thrombosis-online.com). The levels of the CCR5 soluble ligands, RANTES and MIP-1α, were associated with the number of CCR5⁺ intermediate monocytes and CCR2⁺ *patrolling* monocytes (r=0.46; p=0.03 and r=0.51; p=0.01, respectively) (Suppl. Figure 2C-D, available online at www.thrombosis-online.com). No correlations were found between Framingham score and total CD8⁺ T-cells expressing CCR5⁺, total monocytes counts and classical monocyte counts expressing CCR5⁺ (r=0.227; p=0.335, r=0.135; p=0.529 and r=0.023; p=0.92, respectively). Analysing the relationship between the components of the innate immune system revealed that the absolute number of total monocytes was inversely associated with the MFI of CD4 in the mDCs and positively associated with the PDL-1 in the CD1c⁺ mDCs (r=-0.43; p=0.02 and r=0.38; p=0.04, respectively) (Suppl. Figure 2E-F, available online at www.thrombosis-online.com). However, most of these associations were lost after stratification by group probably due to the small number of studied cases (data not shown).

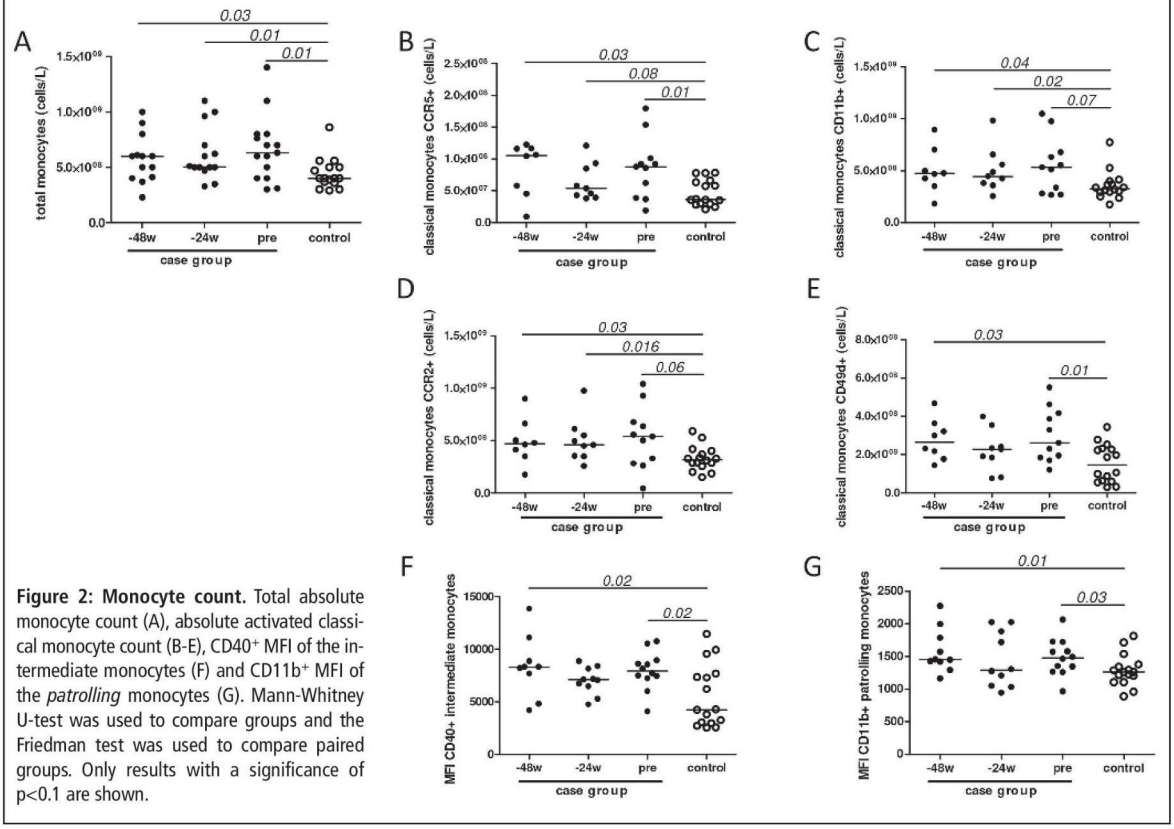

Figure 2: Monocyte count. Total absolute monocyte count (A), absolute activated classical monocyte count (B-E), CD40$^+$ MFI of the intermediate monocytes (F) and CD11b$^+$ MFI of the *patrolling* monocytes (G). Mann-Whitney U-test was used to compare groups and the Friedman test was used to compare paired groups. Only results with a significance of $p<0.1$ are shown.

High neutrophil counts in patients with ACS during the 48 weeks before the event

We observed a borderline statistical significance towards a higher number of neutrophils in the Case group at 48 weeks, 24 weeks and immediately before the ACS (►Figure 4A) compared with the Control group. In all the participants, the number of neutrophils was positively associated with the levels of CCR5$^+$CD8 T-cell (r=0.44; p=0.03) (►Figure 4B), the total number of monocytes (r=0.59; p<0.001) (►Figure 4C) and the levels of CCR5$^+$ classical monocytes (r=0.61; p<0.001) (►Figure 4D). The last two associations were also found at different time-points in the study in only the Case group after segregating by group (data not shown).

IFNα production by pDCs after TLR-9-specific stimulation was reduced in patients with ACS

Similar to mDCs, circulating plasmacytoid dendritic cells (pDCs) are significantly reduced in patients with ACS (13). In that scenario, pDCs may also migrate into lymphoid tissues in response to systemic inflammatory activation where IFN-α production contributes to plaque instability (14). Although no differences were obtained when peripheral pDCs were analysed (Suppl. Table 4, available online at www.thrombosis-online.com), we also studied the functionality of these cells by assessing IFN-α production after TLR-9-specific stimulation of PBMCs. We observed a borderline statistical significance in lower IFN-α production in the Case group at the *pre* time-point compared with the Controls group (Suppl. Figure 3A, available online at www.thrombosis-online.com). As previously described (15), peripheral pDCs and total CD4 T-cells positively correlated with IFN-α production (r=0.41; p=0.05 and r=0.58; p=0.008, respectively). Interestingly, a strong and inverse association was found between IFN-α production and the percentage of total CD8 and CCR5+ CD4 central memory T-cells (r=-0.52; p=0.01 and r=-0.59; *p*=0.003, respectively) (Suppl. Figure 3B-E, available online at www.thrombosis-online.com), suggesting altered functionality of peripheral pDCs in a proinflammatory state.

Discussion

Our observational study comprehensively and longitudinally shows that high expression of activation markers and progressive

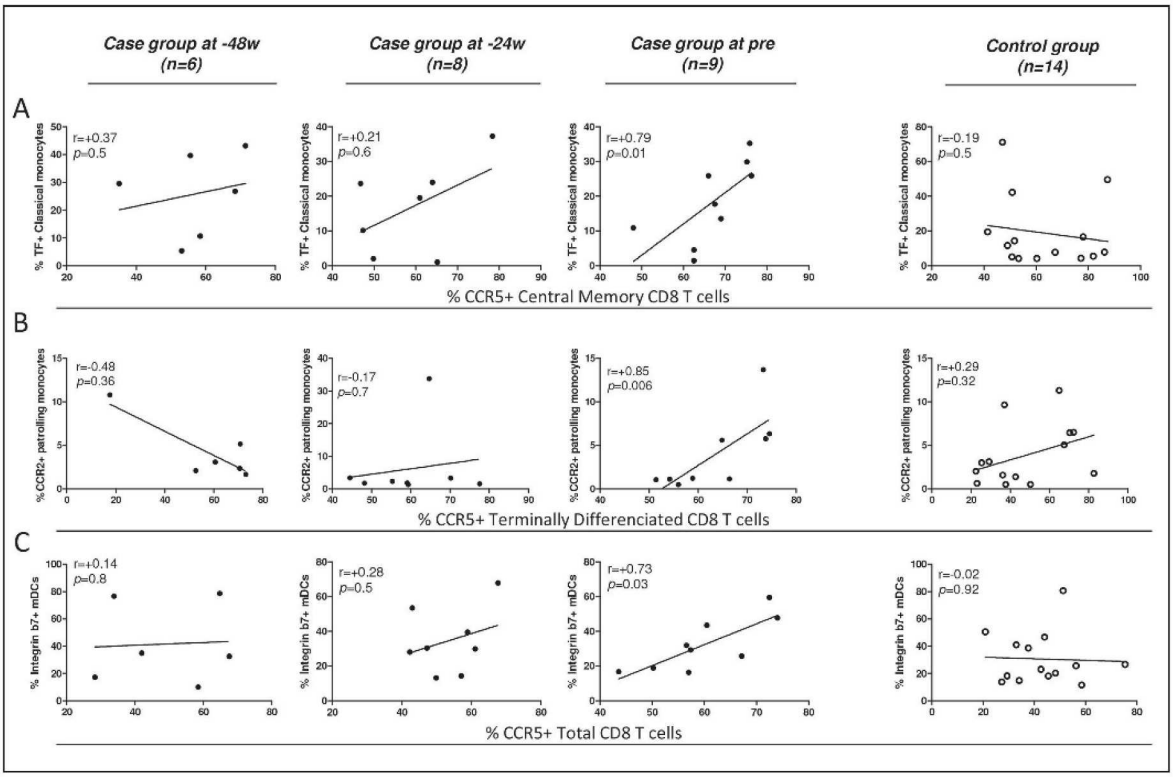

Figure 3: CCR5⁺ CD8 T-cells correlations with monocytes and myeloid dendritic cells in the Case and Control groups. The Case group was studied at 48 weeks, 24 weeks and at the pre time-point (4, [1–7] weeks). The association between tissue factor expression on classical monocytes and CCR2 on patrolling monocytes (A-B) with integrin-β7 on mDCs (C). The Spearman ρ correlation coefficient test was used.

association in components from innate and adaptive immune systems precede the onset of ACS in HIV-infected patients on suppressive cART.

To our knowledge, this is the first longitudinal study showing increased CCR5⁺ CD8 T-cell levels in HIV-infected patients prior to the occurrence of ACS. Previous studies have also analysed T-cell activation in relation to CVD but using surrogate end points such as IMT and other T-cell activation markers (10). A recent study in a large HIV cohort (16) found an association between the number of CD8 T-cells and ACS using clinical end points; however, they did not analyse the phenotypes of the T-cells and their association with the DCs and monocytes. Regarding the monocytes, in this study, we observed an increase in the expression of activation markers (CD11b and CX3CR1) on monocytes, which is in agreement with previous studies (11). In addition, we also observed high levels of other activation and cell adhesion markers, CCR5, CCR2 and CD49d, whose involvement in CVD progression in HIV-infected patients remains unknown. Interestingly, these markers were increased for at least one year prior to the event in the Case group compared with the Control group.

We have found a direct association between activated CD8 T-cell and myeloid cell levels, particularly in monocytes expressing activation and cell adhesion surface markers. These associations were only statistically significant in the group of patients with ACS and in the follow-up time point just before the ACS. These data reflect that the subclinical systemic inflammation state, that defines HIV-infected patients on suppressive cART, can eventually drive to an acute process, leading to the clinical manifestation as ACS. In that context, innate (monocytes, neutrophils and dendritic cells (17–19)) and adaptive (16) immune cells are recruited to the inflammatory focus and contribute to plaque instability through soluble mediators. For example, neutrophils, whose levels also tended to be higher before the event, can trigger T-cell recruitment to plaques through IL1β production in macrophages (20). This environment enriched in proinflammatory cytokine signalling (20) induces defects in antigen presenting cell properties, similar to what we observed in pDCs. These defects may induce aberrant production of lytic granules in effector CCR5⁺CD8 T-cells, chemokines (RANTES, MIP-1α and MIP-1β) and upregulation of their receptors, particularly CCR5, CCR2 and CX3CR1 (21, 22), which fuels T-cell recruitment and eventually leads to endothelial lesion including plaque instability and ACS (schematic diagram in Suppl. Figure 4, available online at www.thrombosis-online.com).

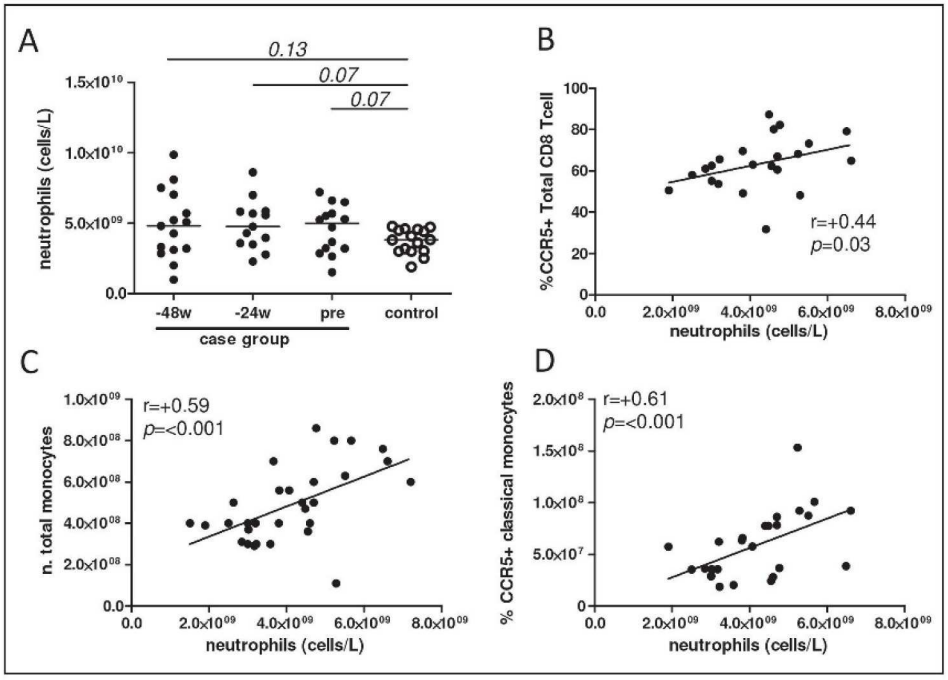

Figure 4: Neutrophil counts and association with T-cell and monocyte activation. Neutrophil counts (A) and correlation with CCR5⁺ expression on total CD8 T-cells (B), the number of total monocytes (C) and correlation between CCR5⁺ expression on classical monocytes with neutrophil counts (D) in all the participants. The Mann–Whitney U and chi-square tests were used to compare groups and the Friedman test was used to compare paired groups. The Spearman ⍰ correlation coefficient test was used. Only results with a significance of p<0.15 are shown.

One question that still remains in this case control study relates to the factor(s) triggering of higher levels of inflammation in the Case group. Apart from HCV coinfection (23) and traditional risk factors, additional coinfections can lead to increased immune activation and CVD. In this sense, CMV coinfection has also been associated with clinical CVD (24); however, all patients analysed were anti-CMV⁺ based on the geographical area, although anti-CMV titres were unknown and infections with other herpes viruses or microorganisms could be playing a significant role.

Among the results presented, this study highlights the important role of CCR5 in the onset of ACS. These results are in agreement with recent evidences supporting the idea that CCR5 and its ligands are also involved in the progression of atherosclerosis and other disorders and can be therapeutically targeted (25–27). In this sense, CCR5Δ32 deletion, besides being a resistant factor for HIV-1 infection in homozygosis, has been associated with a reduced risk for severe coronary artery disease in the general population (28). Additionally, CCR5 antagonists, apart from controlling HIV infection, also have important immunomodulatory effects on homeostasis (29–31). Regarding inflammatory diseases, Martin-Blondel et al. (25) hypothesises that CCR5 antagonists might prevent the deleterious inflammatory reaction that occurs during immune recovery in progressive multifocal leukoencephalopathy patients by interfering with the activation and/or migration of CCR5-expressing activated CD8⁺ T-cells. Regarding plaque formation, it has also been shown that therapeutic use of the CCR5 antagonist, Maraviroc (MVC), reduced plaque by reversing the proinflammatory profile in atherosclerotic ApoE$^{-/-}$ mice (7). It is interesting to note that in our study, no differences were found in the frequency of heterozygotic CCR5Δ32 deletion and cART including CCR5 antagonists (data not shown).

This study has some limitations. First, this study has a small number of participants. This scarcity is explained and counterbalanced by a case control study design, which allowed us to carry out, for the first time to our knowledge, a comprehensive study of the innate and adaptive immune system components in relation to ACS. Further investigations should determine if the findings presented herein could be considered as complementary predictors of CVDs in controlled large cohort studies. In addition, other works should clarify if these results can also be applied to other types of cardiovascular events and clinical scenarios such as stable plaques and to non-HIV-infected population. Because of the retrospective nature of this study, we did not have access to atherosclerotic arterial tissue. In addition, the inclusion of non-HIV-infected population, not bias for clinical assistance and/or hospitalization, was inaccessible in our clinical setting. The results obtained in peripheral blood may mirror the activated state in atherosclerotic plaques. Future studies should demonstrate the association between our findings and arterial tissue damage.

In summary, activated monocytes and CD8 T-cells precede the onset of ACS in HIV-infected patients. In these patients, the association between the innate and adaptive immune systems reveals a progressive homeostatic dysregulation that involves activation of surface markers such as CCR5. Further investigations should determine if the modulation of activated monocytes and CD8 T-cells has clinical implications for mitigating the develop-

What is known about this topic?

- T-cell activation has been associated with carotid intima-medial thickness (IMT) as a surrogate marker of vascular disease in HIV-infected patients in cross-sectional studies. Similar results have been found for monocyte activation.
- However, other works have found controversial results regarding these associations, showing no association in T-cell and monocyte activation with the development of cardiovascular pathologies.
- Bulk CD8 T-cell count, but not a detailed phenotype, has been associated with a clinical endpoint, increased acute myocardial infaction risk, in male HIV-infected subjects.

What does this paper add?

- Our observational study comprehensively and longitudinally shows that high CCR5$^+$ CD8$^+$ T-cell and monocyte activation levels precede the onset of acute coronary syndrome (ACS) in HIV-infected patients on suppressive combined antiretroviral therapy (cART).
- The interplay between innate and adaptive immune system components is shown by a progressive association in activation of monocytes and CD8$^+$T-cells is only shown in patients who are going to suffer the ACS.
- Important role of CCR5 and monocyte activation as markers of cardiovascular risk and potential therapeutic targets in HIV-infected patients.

Abbreviations

cART: combined antiretroviral therapy, CVD: cardiovascular disease, ACS: acute coronary syndrome, IMT: intima-medial thickness, hsCRP: high-sensitivity C reactive protein, CMV: cytomegalovirus, MFI: median fluorescence intensity, DCs: dendritic cells, TF: tissue factor, mDCs: myeloid dendritic cells, pDCs: plasmacytoid dendritic cells, MVC: Maraviroc, PBMC: peripheral blood mononuclear cell, HCV: hepatitis C virus.

ment of cardiovascular disease, one of the main causes of morbidity and mortality in HIV-infected patients.

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Author contributions

L.T.-D., E.R.-M. and M.L. designed the study and M.G. and M.L. coordinated the patients inclusion. E.R.-M, L.T.-D., R.P.-B., I.R.-S. and B.D.-M. designed the experiments. L.T.-D., R.P.-B., AI.A.-R. and JL.J. produced the experimental data in the laboratory. L.T.-D. and R.P.-B. analysed the data. L.T.-D. and R.P.-B. prepared the manuscript. YM.P., M.A.M.-F., E.R.-M. and M.L. contributed to reviewing the manuscript.

Conflicts of interest

None declared.

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Role of TLR4 Asp299Gly polymorphism in the development of cardiovascular diseases in HIV-infected patients

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Role of toll-like receptor 4 Asp299Gly polymorphism in the development of cardiovascular diseases in HIV-infected patients

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Objective: Cardiovascular diseases (CVDs) are one of the main causes of morbimortality in HIV-infected patients on suppressive antiretroviral therapy. The objective of this work was to evaluate the role of single nucleotide polymorphisms (SNPs) in lipopolysaccharide (LPS) Toll-like receptor 4 (TLR4) and CVDs occurrence in HIV-infected patients. Additionally, the functional consequences of carrying these SNPs were analyzed.

Methods: The association of TLR4 SNPs, Asp299Gly/Thr399Ile with CVDs occurrence was analyzed using multivariate logistic regression models. Clinical, immunological, and traditional cardiovascular risk factors were used as covariates. The monocyte phenotype and response were assessed by multiparametric flow cytometry comparing carriers with noncarriers of this SNP.

Results: Asp299Gly SNP, assayed in 253 HIV-infected patients, was independently associated with the occurrence of CVDs after adjusting for $CD4^+$ T-cell nadir, HCV-coinfection, bacterial pneumonia, diabetes mellitus, and traditional cardiovascular risk factors [odds ratio (confidence interval 95%) = 3.672 (1.061–12.712), $P=0.04$). Carriers of Asp299Gly SNP showed higher percentage of patrolling and intermediate monocytes producing a proinflammatory combination of cytokines compared with noncarriers ($P=0.037$ and $P=0.046$, respectively). Intermediate monocyte subset levels correlated with soluble interleukin-6 levels only in carriers ($r=0.89$; $P=0.01$).

Conclusion: TLR4 Asp299Gly polymorphism is independently associated with the occurrence of CVDs in HIV-infected patients. The proinflammatory profile associated to this variant could be involved in the development of atherosclerotic pathologies.

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Introduction

The AIDS-related morbidity and mortality has undoubtedly been reduced due to the introduction of combined antiretroviral therapy (cART). Nevertheless, non-AIDS-related events, such as those attributable to cardiovascular diseases (CVDs), are increased approximately two-fold compared to non-HIV-infected population [1], being nowadays one of the main causes of morbidity and mortality in HIV-infected-patients [2]. The interplay between traditional CVD risk factors [3] and the effect of some type of antiretroviral therapies [4] have been traditionally associated with the higher prevalence of CVDs; however, more recent studies have pointed toward the importance of inflammation and activation of the adaptive and innate immune systems [5,6] as another main source of these pathologies. Toll-like receptor 4 (TLR4) is a pathogen-associated molecular pattern receptor of bacterial lipopolysaccharide (LPS). This receptor is responsible for monocyte, macrophages, and endothelial cells activation [7] and is expressed in human atherosclerotic lesions [8]. Multiple single nucleotide polymorphisms (SNPs) have been identified in the human *TLR4* gene, of them, Asp299Gly (rs4986790) and Thr399Ile (rs4986791) SNPs, that are in strong linkage disequilibrium, have been related with the risk of cardiovascular events [9,10], and factors of the metabolic syndrome [11,12] in non-HIV-infected individuals; however, some other studies have not found this association [13,14].

In the HIV-infection scenario, studies to evaluate the contribution of Asp299Gly and Thr399Ile SNPs in the pathogenesis of HIV infection are limited but suggest a higher AIDS progression and susceptibility to serious infections on carriers of these polymorphisms [15,16]. However, the role of TLR4 Asp299Gly and Thr399Ile polymorphisms on the development of CVDs in HIV-infected patients has not been explored.

Therefore, the aim of the present study was to analyze the whether TLR4 Asp299Gly SNP was independently associated with the occurrence of CVDs in HIV-infected patients and to investigate the functional consequences of carrying this polymorphism.

Methods

Study participants

A total of 253 HIV-infected patients who consecutively visited the Virgen del Rocío University Hospital in Seville (Spain) between 1989 and 2016 were included in the study. The selection criteria were as previously published [17]. Briefly, to have frozen peripheral blood mononuclear cell samples available for genotyping; secondly, to belong to white ethnic group; and, to have immunological, virological, and clinical data recorded, including non-AIDS-event-related information, during the follow-up. Including records of atherosclerotic CVD (i.e. coronary heart diseases, severe arrhythmia requiring hospitalization and pacemaker, cerebrovascular diseases, sudden death, and mesenteric ischemia).

Laboratory determinations

Laboratory evaluations were performed at the Laboratory of Immunovirology, Institute of Biomedicine of Seville (IBiS) at Virgen del Rocío University Hospital in Seville (Spain) and at Molecular Immunobiology Laboratory, Gregorio Marañon General Universitary Hospital, Madrid (Spain). Institutional and ethical review board approvals were obtained (PI16/00684) and written informed consent was obtained from all study patients.

Total DNA was extracted from peripheral blood mononuclear cell samples using the MagNa Pure LC DNA isolation kit. PCR conditions, concentrations, and determinations of the Asp299Gly and Thr399Ile TLR4 polymorphisms were performed as described previously [17]. Genotypes were determined using the LightCycler 480 System (Roche Diagnostics, Barcelona, Spain) and detection probes for the polymorphisms were designed using the LightCycler Probe Design Software 2.0.

Absolute monocyte, $CD4^+$, and $CD8^+$ T-cell counts (cells/μl) were determined using an Epics XL-MCL flow cytometer (Beckman-Coulter, Brea, California, USA) according to the manufacturer's instructions. Sera and plasma samples were collected and stored at -20°C until subsequent analysis. HCV-RNA (hepatitis C virus) was determined on sera samples using an available PCR procedure kit (COBAS Amplicor, Roche Diagnosis) with a detection limit of 10 IU/ml. HCV exposure (measured by testing for the presence of anti-HCV) was detected using an HCV-specific ELISA (Siemens Healthcare Diagnosis, Malvern, Pennsylvania, USA). High-sensitivity C-reactive protein (hsCRP) and β_2-microglobulin were determined on sera with an immunoturbidimetric assay using COBAS 701 (Roche Diagnostics, GmbH, Mannheim, Germany). D-dimer levels were determined using an automated latex enhanced immunoassay (HemosIL, D-Dimer HS 500; Instrumentation Laboratory) in plasma samples. Soluble interleukin-6 was measured in plasma by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, Minnesota, USA).

Immunophenotyping and intracellular cytokine staining of monocytes

Immunophenotyping and intracellular cytokine staining of monocytes were performed as previously described [18] in a group of 40 healthy donors. Briefly, 1 ml peripheral fresh whole blood samples were collected in EDTA tubes, erythrocytes were then lysed and cells were immediately stained using a panel of antibodies for lineage, activation, cell adhesion surface markers, and

viability dye to exclude nonviable cells. For intracellular cytokine staining, the cells were stimulated with 1 ng/ml LPS for in-vitro stimulation or without stimuli for ex-vivo results in the presence of 1 μl/ml of anti-$CD28^+$ and anti-CD49d (BD Biosciences, Franklin Lakes, New Jersey, USA) and 10 μg/ml of Brefeldin A (Biolegend, San Diego, California, USA) at 37°C/5% CO_2 for 6 h. Monocytes subsets were defined as high Forward/Side scatter and expressed human leukocyte antigen-DR^+, $CD14^+$, and/or $CD16^+$ but not $CD3^+$, $CD8^+$, $CD19^+$, or $CD56^+$ following a gating strategy that can be seen in Supplementary Figure 1, http://links.lww.com/QAD/B243, as previously described [18]: the classical monocytes ($CD14^{++}CD16^-$), the intermediate monocytes ($CD14^{++}CD16^+$), and the nonclassical or patrolling monocytes ($CD14^+CD16^+$) were defined. Surface and intracellular staining for cytokine production of IL1α, IL1β, IL6, IL8, IL10, and tumor necrosis factor (TNF)α was then performed. Isotype controls were included in each experiment and the responsiveness of in-vitro cells was calculated by subtracting the results of the unstimulated condition. Multiparametric flow cytometry was done using an LSR Fortessa Cell Analyzer (BD Biosciences) and a minimum of 2 000 000 total events and 20 000 monocytes were recorded in each tube. Data were analyzed using Flowjo 8.7.7 (TreeStar, San Carlos, California, USA).

Statistical analysis

Continuous variables were expressed as medians and interquartile ranges (IQR), and categorical variables were expressed as number and percentages. Correlations between variables were assessed using Spearman's rank test. The Mann–Whitney *U* test was used to analyze differences between groups. All *P* values <0.05 were considered significant. A multivariate logistic regression model was used to determine the factors associated with the occurrence of CVDs. Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS 22.0; SPSS, Chicago, Illinois, USA). The percentage of monocytes that produce a certain combination of cytokines and the generation of graphs were determined using Pestle, version 1.6.2, Spice, version 5.2 (both provided by M. Roederer; NIH, Bethesda, Maryland, Inc., La Jolla, California, USA).

Results

Characteristics of the studied individuals

General characteristics of all participants are presented in Table 1. Twenty-six HIV-infected patients presented CVD (10%). Cardiovascular events observed were (*n* (%)): coronary heart diseases, 16 (61%), severe arrhythmia requiring hospitalization, four (15%), cerebrovascular diseases, three (12%), sudden death, two (8%), and mesenteric ischemia, one (4%). This group with CVD presented statistically higher proportion of HCV-coinfected patients, total cholesterol and triglycerides levels, and a trend toward higher frequency of TLR4 Asp299Gly SNP carriers compared to the group with no CVD. Regarding both studied TLR4 Asp299Gly SNPs (Asp299Gly and Thr399Ile), we found a 98.8% co-segregation level between both in all studied patients, and hence in advance, results are only presented for Asp299Gly SNP. In addition, patients with CVD had lower $CD4^+$ T-cell nadir compared with patients without CVDs. No differences were observed between groups comparing the traditional cardiovascular risk factors: hypertension, smoking, and diabetes mellitus.

The toll-like receptor 4 single nucleotide polymorphism (Asp299Gly) was independently associated with the occurrence of cardiovascular diseases

In order to determine whether the abovementioned factors were independently associated with CVD

Table 1. Patients' characteristics.

	All patients (*n* = 253)	Patients with CVD (*n* = 26)	Patients without CVD (*n* = 227)	*P*
Age at HIV diagnosis (years)	29 [25–35]	29 [28–34]	29 [25–36]	0.390
Follow-up[a] (years)	19 [12–25]	18 [13–32]	19 [12–25]	0.357
Male sex, *n* (%)	191 (75)	21 (80)	170 (75)	0.509
IDU, *n* (%)	123 (49)	14 (54)	109 (48)	0.573
AIDS, *n* (%)	87 (34)	12 (46)	75 (33)	0.182
Nadir $CD4^+$ T-cell (cells/μl)	186 [67–286]	140 [29–224]	187 [70–291]	0.067
HCV RNA detected, *n* (%)	113 (45)	18 (69)	95 (42)	0.008
Bacterial pneumonia, *n* (%)	51 (20)	8 (31)	43 (19)	0.154
Diabetes mellitus, *n* (%)	22 (9)	4 (15)	18 (8)	0.201
Hypertension, *n* (%)	23 (9)	3 (11)	20 (9)	0.647
Smoking, *n* (%)	103 (41)	13 (50)	90 (40)	0.309
Cholesterol (mg/dl)	182 [152–216]	198 [174–230]	178 [151–214]	0.024
Triglicerides (mg/dl)	124 [87–183]	182 [130–291]	115 [84–175]	0.001
TLR4 Asp299Gly, *n* (%)	32 (13)	6 (23)	26 (11)	0.091

Continuous variables are expressed as the median and interquartile ranges (IQR). Categorical variables are expressed as number and percentages. The Mann–Whitney *U* test and chi-square tests were used. cART , combined antiretroviral therapy; CI, confidence interval; CVD, cardiovascular disease; HCV, hepatitis C virus; OR, odds ratio; TLR4, toll-like receptor 4.
[a]During the follow-up, 96% of the individuals were on cART.

Table 2. Factors associated with cardiovascular disease.

	P; OR (95%) Univariate	*P*; OR (95%) Multivariate
Age at HIV diagnosis (years)	0.973; 0.99 (0.96–1.04)	
Follow-up (years)	0.535; 0.98 (0.94–1.03)	
Male sex, *n* (%)	0.511; 1.4 (0.51–3.91)	
IDU, *n* (%)	0.574; 1.26 (0.56–2.85)	
AIDS, *n* (%)	0.186; 1.74 (0.77–3.94)	
Nadir $CD4^+$ T-cell (cells/μl)	**0.054; 0.99 (0.99–1)**	0.092; 0.996 (0.991–1.001)
HCV RNA detected, *n* (%)	**0.011; 3.13 (1.3–7.49)**	0.064; 2.59 (0.945–7.12)
Bacterial pneumonia, *n* (%)	0.16; 1.9 (0.78–4.66)	0.21; 2.016 (0.674–6.025)
Diabetes mellitus, *n* (%)	0.21; 2.11 (0.66–6.8)	0.072; 3.567 (0.894–14.227)
Hypertension, *n* (%)	0.648; 1.35 (0.37–4.89)	
Smoking, *n* (%)	0.311; 1.52 (0.675–3.434)	
Cholesterol (mg/dl)	**0.024; 1.01 (1.01–1.02)**	**0.036; 1.011 (1.001–1.022)**
Tryglicerides (mg/dl)	**0.001; 1.01 (1.01–1.02)**	**0.01; 1.005 (1.001–1.009)**
TLR4 Asp299Gly, *n* (%)	**0.099; 2.31 (0.85–6.30)**	**0.04; 3.672 (1.061–12.712)**

Multivariate logistic regression model was performed. Variables with *P* values <0.1 in the unadjusted model and variables biologically associated with the occurrence of CVDs were included in the multivariate analysis (in bold), *P* values <0.05 in the adjusted model are considered as statistically significant (in bold). CI, confidence interval; CVD, cardiovascular disease; HCV, hepatitis C virus; OR, odds ratio; TLR4, toll-like receptor 4.

occurrence, we performed a multivariate logistic regression, showed in Table 2. Variables with *P* values <0.1 in the unadjusted model and variables biologically associated with the occurrence of CVDs were included in the multivariate analysis. After adjusting for $CD4^+$ T-cell nadir, HCV-coinfection, bacterial pneumonia, diabetes mellitus, cholesterol and triglyceride levels, and TLR4 Asp299Gly SNP, the factors associated with the occurrence of CVDs were cholesterol, triglycerides, and, interestingly, the TLR4 Asp299Gly SNP [odds ratio (OR) (confidence interval [CI 95%]) = 1.011 (1.001–1.022), $P = 0.036$; OR (CI 95%) = 1.005 (1.001–1.009, $P = 0.01$ and OR (CI 95%) = 3.672 (1.061–12.712), $P = 0.04$, respectively]. The association of HCV-coinfection, diabetes mellitus, and $CD4^+$ T-cell nadir remained in a trend ($P < 0.1$).

In-vitro intracellular cytokine production of monocyte subsets was higher in individuals carrying toll-like receptor 4 Asp299Gly single nucleotide polymorphism compared with noncarriers

We performed a comprehensive study of monocyte phenotype and functionality in a set of healthy donnors fresh samples. Individuals carrying TLR4 Asp299Gly and noncarriers were matched by age, sex, $CD4^+$ and $CD8^+$ T-cell counts, monocyte counts and hsCRP, β_2-microglobuline and D-dimer levels. No differences among the different variables were found between carriers and noncarriers. For this reason, for comparison, no multiple corrections were performed (Supplementary Table 1, http://links.lww.com/QAD/B243). We analyzed the ex-vivo and in-vitro (in response to LPS) intracellular cytokine production of monocytes producing simultaneously up to six cytokines, also termed polyfunctionality, after background substraction of the unstimulated condition. Analyzing the 63 multiple cytokine combinations *in vitro*, a higher percentage of patrolling monocytes expressing the combination $IL1\alpha^+IL1\beta^-IL6^-IL8^+IL10^-TNF\alpha^-$ and intermediate monocytes producing the proinflammatory combination of cytokines $IL1\alpha^-IL1\beta^-IL6^+IL8^-IL10^-TNF\alpha^-$ in individuals carrying the TLR4 Asp299Gly SNP comparing with noncarrier individuals was found ($P = 0.037$ and $P = 0.046$, respectively) (Fig. 1 a and b). No significant combination of cytokines was differentially produced in classical monocyte subset. We also determined the soluble plasma concentration of the inflammatory biomarker, interleukin-6. Soluble interleukin-6 levels were positively and strongly correlated with percentage of intermediate monocytes expressing the combination $IL1\alpha^-IL1\beta^-IL6^+IL8^-IL10^-TNF\alpha^-$ only in individuals with Asp299Gly allele ($r = 0.89$; $P = 0.01$) but not in wild type individuals (Fig. 1 c and d).

Finally, we did not find differences in the frequency of monocyte subsets comparing TLR4 Asp299Gly SNP carriers and noncarriers. The percentage of activation and cell adhesion markers $CD11b^+$, $CD40^+$, $CD49d^+$, $CD62L^+$, $CD163^+$, and $CD38^+$ were also similar regarding the three monocyte subsets (data not shown). No differences were observed for ex-vivo single cytokine production and polyfunctionality distribution between TLR4 Asp299Gly SNP carriers and noncarriers (data not shown). All these experiments were also performed in a small subset of HIV-infected patients with fresh samples available and similar results were found in the intermediate monocyte subset. Carriers produced combinations of cytokines including $IL8^+$ and $IL10^+$ at higher levels than noncarriers ($P < 0.05$).

Discussion

In this study we have identified TLR4 Asp299Gly SNP as a factor independently associated with the development of CVDs in a well characterized cohort of HIV-infected patients. Additionally, we observed a proinflammatory

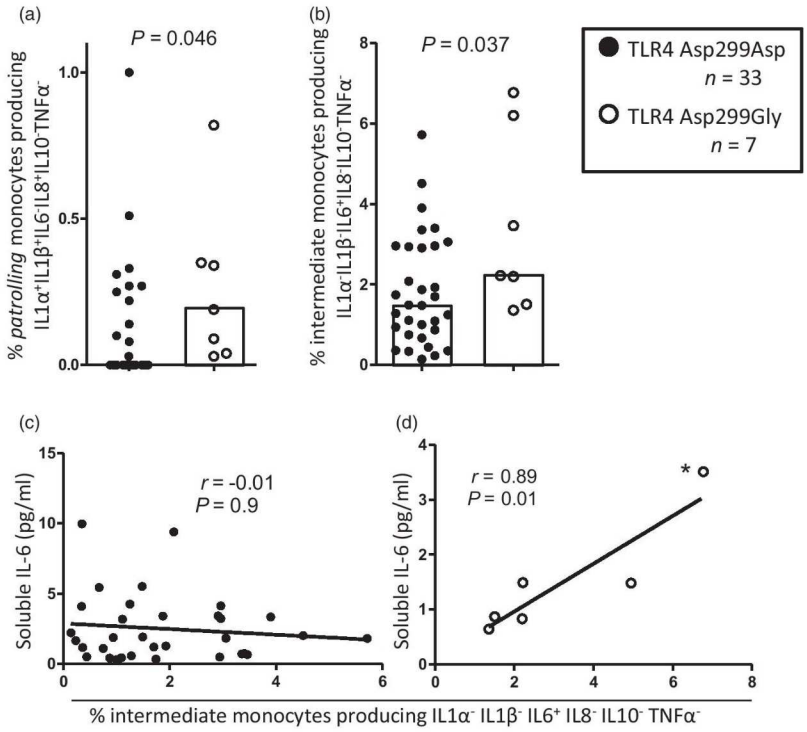

Fig. 1. Cytokine combinations produced by monocyte subsets in response to lipopolysaccharide (LPS) in healthy donors. Percentage of patrolling monocytes producing the combination IL1α$^+$IL1β$^-$IL6$^-$IL8$^+$IL10$^-$TNFα$^-$ (a) and intermediate monocytes producing the combination of cytokines IL1α$^-$IL1β$^-$IL6$^+$IL8$^-$IL10$^-$TNFα$^-$ (b) between groups in response to in-vitro stimulation with LPS. Correlation of soluble interleukin-6 and percentage of intermediate monocytes producing the combination of cytokines IL1α$^-$IL1β$^-$IL6$^+$IL8$^-$IL10$^-$TNFα$^-$ on individuals with toll-like receptor 4 (TLR4) Asp299Asp single nucleotide polymorphism (SNP) (c), and with TLR4 Asp299Gly SNP (d). The statistical significant correlation remained after excluding the outlier value (*) ($r = 0.88$; $P = 0.03$). Pestle, version 1.6.2, and Spice, version 5.2 were used for analysis. Mann–Whitney *U* test was used to compare groups.

profile in the innate immune system that could be involved in the development of these pathologies.

TLR4, as part of the innate immunity response cascade, was identified as one of the factors contributing to the deleterious process of atherosclerosis [19]. In a recent study based on proteomic profile in plasma and public genome-wide association data, TLR4 has been proposed indirectly to be involved in a mechanistic path associated with CVDs [20]. The Asp299Gly SNP is known to influence TLR4 signaling and function [21]; however, the impact of this variant on CVDs in general population remains still controversial, being associated with CVDs only in few studies [9], whereas others did not find this association [14]. One of the reasons of those discrepancies could be that most of the related studies used intima–media thickness as surrogate end-point [22]. The present finding in HIV-infected patients is the first identifying TLR4 Asp299Gly SNP as a risk factor using a clinical end-point instead of traditional surrogate markers as intima–media thickness.

Apart from TLR4 SNP association, the results presented herein also showed total cholesterol and triglyceride levels independently associated with the occurrence of these pathologies. Far from looking for immunological CVD predictors alternative to major well established CVD risk factors, the main aim of this study was to identify immunological factors associated to CVDs and related mechanisms that could explain the occurrence of that pathologies. Diabetes mellitus frequency was not associated to CVDs in our study. Although remained in a trend, our results are in agreement with recent data from a larger cohort of HIV/HCV-coinfected patients [23]. Our findings also revealed an association between HCV and bacterial infection with the occurrence of CVDs. This is in agreement with previous studies carried out in non-HIV-infected population that showed the

implication of gram-negative bacterial and viral infections in atherosclerosis development and progression [24,25]. Interestingly, some studies have suggested a correlation between TLR4 polymorphisms and susceptibility to infectious diseases such as gram-negative infections due to an impaired innate immune response [26,27].

In addition, we have supported these cohort study results by functional mechanisms in a set of healthy donors. Our group has recently observed the role of activated monocytes in the development CVDs in the HIV-population [5]. In this work, we have analyzed the functional consequences of TLR4 Asp299Gly SNP determining the in vitro monocyte response to LPS compared with noncarriers. *Ex vivo*, no differences were observed in monocyte activation markers; however, after LPS stimulation *in vitro*, individuals with TLR4 Asp299Gly SNP had high level of monocytes producing combinations of proinflammatory cytokines. Those levels were strongly correlated with soluble interleukin-6, cytokine that has been strongly associated in HIV-infection with progression and mortality [28]. This finding points to intermediate monocyte subsets as the main source of soluble interleukin-6 in agreement with our previous results [29]. Both, intermediate and patrolling subsets, have been shown to be involved in monocyte crawling and transendothelial migration [30], eventually favoring the occurrence of CVDs [31]. These results support and extend those of a previous work [32] and differed with other previous studies that observed a cytokine expression hyporesponsiveness [33,34] may be due to the current geographical distribution of TLR4 haplotypes in Europe that differs from Africa [35] and due to the in-depth qualitative and quantitative analyses of combinations of up to six cytokines produced in monocyte subsets, being the first work carried out with such a comprehensive approach *ex vivo* and *in vitro* based on TLR4 SNP.

This work has some limitations. The relative scarcity number of recorded cardiovascular events is counter-balanced by the exhaustive follow-up of HIV-infected patients. Because of the retrospective nature of this study, we did not have access to the atherosclerotic arterial tissue. The results obtained in peripheral blood may mirror the activated state in atherosclerotic plaques. Future studies should demonstrate the association between our findings and arterial tissue damage and if our results can also be applied to other types of clinical manifestations like stable plaques.

In summary, TLR4 Asp299Gly SNP is associated with CVDs in HIV-infected patients. The proinflammatory monocyte profile associated with this polymorphism could explain, together with other mechanisms, the proatherogenic environment and the development of atherosclerotic pathologies.

Conclusion

TLR4 Asp299Gly SNP is a factor independently associated with the development of CVDs in a well characterized cohort of HIV-infected patients. The proinflammatory profile associated to this variant could be involved in the development of atherosclerotic pathologies.

Acknowledgements

Authors' contributions: L.T.-D., E.R.-M., and M.L. designed the study and M.G. and M.L. coordinated the patients inclusion. E.R.-M, L.T.-D., R.P.-B., and I.R.-S. designed the experiments. L.T.-D., R.P.-B., AI.A.-R., and J.L.J. produced the experimental data in the laboratory. L.T.-D. and R.P.-B. analyzed the data. L.T.-D. prepared the manuscript. M.A.M.-F., E.R.-M., and M.L. contributed to reviewing the manuscript.

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Conflicts of interest

There are no conflicts of interest.

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Hepatitis C virus and cumulative infections are associated with atherogenic cardiovascular events in HIV-infected subjects

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ABSTRACT

Objectives: to analyze the association between HCV coinfection and cumulative infections with the development of a cardiovascular disease in HIV-infected subjects.
Methods: HIV-infected subjects attended at Virgen del Rocio University Hospital, between January 1982 and March 2018, were considered if fulfilled the following criteria: at least two visits to the HIV clinic, clinical records with data about VZV reactivation and bacterial infections, available data on HCV coinfection status. Atherogenic cardiovascular events were registered. To analyze factors associated with the development of cardiovascular event, a logistic regression analysis was performed.
Results: 823 subjects were included in the study. During the observational period, 58/823 (7.05%) developed a cardiovascular event. Advanced age at HIV-1 diagnosis, a low T-CD4 nadir, HCV coinfection and the burden of infections were independently associated with the risk of developing a cardiovascular event, apart from lipid levels and diabetes.
Conclusions: both HCV and the burden of infections are associated with an increased risk of cardivascular event in HIV-infected patients, together with other cardiovascular risk factors. Therapeutic strategies such as HCV erradication or VZV immunization could ameliorate cardiovascular risk in these subjects.

1. Introduction

Cardiovascular disease (CVD) is currently the leading cause of death and premature disability in developed societies (Roger et al., 2012). Apart from classical cardiovascular risk factors, both acute and chronic infections have been independently associated with higher risk of developing cardiovascular events and with high levels of different atherosclerosis surrogate biomarkers (Arcari et al., 2005; Emsley et al., 2008; Kozarov et al., 2015; Warren-Gash et al., 2009), in HIV uninfected subjects.

Regarding chronic infections, CVD has emerged as a one of the main causes of morbidity and mortality among HIV-infected subjects, including those on effective combined antiretroviral therapy (cART), being considered HIV-1 itself as a major cardiovascular risk factor (Freiberg et al., 2013; Nou et al., 2016). The role of hepatitis C virus (HCV) over the cardiovascular risk has also been analyzed, but discordant results were found among different authors (Arcari et al., 2006; Oliveira et al., 2013; Wong et al., 2014). This issue can be currently overcome since high effective direct antiviral agents are now available, achieving HCV eradication on the other of 100% of subjects (Asselah et al., 2018). In addition, an increased risk of CVD with herpes zoster (VZV) reactivation, community acquired pneumonia, influenza virus

Abbrevation: CVD, cardiovascular disease; cART, combined antiretroviral therapy; HCV, hepatitis C virus; VZV, herpes zoster virus; IQR, interquartile range

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Table 1
Baseline characteristics of the global population and comparison between those subjects experiencing a CVD and those without CVD during the observation period.

	Global (N = 823)	CVD (N = 58)	No CVD (N = 765)	*P*
Age at diagnosis, years [IQR]	30.5 [24–36]	35 [28–42]	30 [24–36]	**0.002**
Male sex, n (%)	668 (81.2)	50 (86.2)	618 (80.8)	0.385
AIDS, n (%)	209 (25.4)	27 (46.6)	182 (23.8)	**< 0.001**
T-CD4 nadir < 200 cell/μL, n (%)	386 (46.9)	40 (69)	346 (45.2)	**0.001**
IDU[a], n (%)	317 (38.5)	33 (56.9)	284 (37.1)	**0.005**
HIV diagnosis precART[b], n (%)	373 (45.3)	37 (63.8)	336 (43.9)	**0.004**
HCV infection[c], n (%)	297 (36.1)	32 (55.2)	265 (34.6)	**0.003**
Herpes zoster reactivation, n (%)	99 (12)	12 (20.7)	87 (11.4)	0.056
Acute bacterial infection, n (%)	137 (16.6)	15 (25.9)	122 (15.9)	0.066
Diabetes, n (%)	48 (5.8)	11 (19)	37 (4.8)	**< 0.001**
Total cholesterol, mg/dl [IQR]	182 [153–209]	207 [173–230]	181 [152–207]	**< 0.001**
Triglycerides, mg/dl [IQR]	139 [80–166]	202 [110–237]	134 [78–161]	**< 0.001**
Cumulative infections				
None, n (%)	388 (47)	16 (27.6)	371 (48.5)	**0.002**
One, n (%)	302 (36.7)	25 (43.1)	277 (36.2)	**0.003**
≻ 2, n (%)	134 (16.3)	17 (29.3)	117 (15.3)	**< 0.001**

[a] Intravenous drug users.
[b] Diagnosis of HIV before 1996, when combined antiretroviral therapy was not available.
[c] HCV: positive HCV-PCR at any time during the observation period.

and even with cumulative childhood acquired infections has been recently reported (Burgner et al., 2015a,b; Kwong et al., 2018; Minassian et al., 2015; Musher D.M., 2019; Violi et al., 2017). However, the effect of HCV and/or cumulative infections over the risk of developing CVD in the context of HIV-infection has been scarcely explored (Fernández-Montero et al., 2016; Masiá et al., 2011). To explore the role of acute and chronic infections over the cardiovascular risk is relevant in order to better understanding other mechanisms involved in atherogenesis. Moreover, since effective therapy and immunization are currently available for many of these infections, CVD could be prevented through both therapeutic strategies.

Hence, the first and main objective of our study was to analyze the association between HCV coinfection with the development of a CVD in a cohort of HIV-infected subjects. Second, we aimed at analyzing whether the cumulative effect of different infections was associated with CVD in this setting.

2. Materials and methods

2.1. Patients

A retrospective observational study was designed, including 1055 patients that were attended at Virgen del Rocio University Hospital, Sevilla, between January 1982 and March 2018. The patients that fulfilled the following criteria were included in our study: at least two visits to the HIV clinic, clinical records with data about VZV reactivation and bacterial infections, available data on HCV coinfection status (HCV antibodies and qualitative HCV-PCR). These criteria were not available in 232 subjects of the 1055 patients and were excluded. Summarizing, the final study cohort was composed by 823 subjects. The Ethical Committee of the hospital approved the study and all patients signed the written informed consent.

For the purpose of this analysis, HCV infection was considered whether both HCV antibodies and qualitative HCV-PCR were positive. We considered that a patient was uninfected by HCV if the qualitative HCV-PCR was negative, independently the HCV antibodies status. Thus, we excluded patients with spontaneous HCV clearance. However, patients who had achieved sustained virological response after anti-HCV therapy were considered as HCV-infected subjects.

Apart from HCV status, in order to consider the burden of infections, VZV reactivation and acute bacterial infections (community acquired pneumonia, urinary sepsis, Fournier's gangrene) were included if documented in clinical records. Thus, this variable was considered as follows: 0) no coinfections; 1) at least one coinfection (HCV, VZV or bacterial infection); and 2) two or more coinfections. For those subjects developing a CVD, infections were considered if ocurred before the CVD. Once recurrent CVD was developed in some subjects, for the purpose of this analysis the first event was considered.

Cardiovascular events were those with an atherosclerotic origin: acute coronary syndrome, stroke, peripheral arteriopathy and mesenteric ischemia. The lipid profile determination considered in our study was the last one available in clinical records for each subject if no CVD during the observational period was developed. We considered the lipid profile determination available immediately before CVD for those patients developing a CVD.

2.2. Laboratory determinations

Absolute CD4 and CD8 T-cell counts (cells/mm3) were determined using an Epics XL-MCL flow cytometer (Beckman-Coulter, Brea, CA) according to the manufacturer's instructions. HCV-RNA (Hepatitis C virus) was determined on sera samples using an available PCR procedure kit (COBAS Amplicor, Roche Diagnosis) with a detection limit of 10 IU/mL. HCV exposure (measured by testing for the presence of anti-HCV) was detected using a HCV-specific ELISA (Siemens Healthcare Diagnosis, Malvern, Pennsylvania).

2.3. Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS 23.0, Inc, Chicago, USA). All continues variables were expressed as median (interquartile range, IQR), and categorical ones as number of cases (percentage). Differences between groups were analyzed with the Mann-Whitney *U* test for continuous variables, while the chi-square test was used for categorical ones. To analyze the independent factors associated with the development of the first CVD, a bivariate logistic regression analysis was performed and variables with p values < 0.1 in the bivariate analysis were included in a multivariate logistic regression analysis. All differences with $p < 0.05$ were considered statistically significant.

3. Results

Baseline characteristics of the global population and comparison between subjects developing or not a CVD are shown in Table 1. During the observational period, 58 subjects experienced a cardiovascular event (58/823, 7.05%). The most frequent CVD was the acute coronary syndrome, developed by 38 subjects. Median age when subjects

developed CVD was 47 years (IQR: 41–53). Additionally, 14 patients developed recurrent cardiovascular events (14/58, 24.14%), all of them but one expressed as acute coronary syndrome. From 58 patients that experienced a CVD, 21 of them died after developing the cardiovascular event (21/58, 36.21%). Causes of death among these patients were CVD (17/21, 80.95%), cancer (2/21, 9.52%), acute bacterial infection (1/21, 4.76%) and end-stage liver disease (1/21, 4.76%).

Interestingly, 50/58 (86.2%) of these subjects experienced a CVD despite an effective cART, since HIV viral load was persistently undetectable before developing the CVD. Furthermore, other traditional CVD risk factors were scarcely represented in these subjects, since hypercholesterolemia (cholesterol levels > 200 mg/dl) was present in 31/58 patients (53.4%) and only 11/58 subjects (19%) had diabetes mellitus. Taking altogether, 24/58 subjects (41.38%) experienced a CVD with a median age of 47 years (IQR 41–53), despite an effective cART, without diabetes and with normal lipid levels. Among these 24 "low-risk" subjects, 5 (5/24, 20.8%) developed recurrent CVD and 8 (8/24, 33.3%) died after the development of CVD.

When variables potentially associated with the development of a CVD were analyzed, advanced age at HIV-1 diagnosis, a low T-CD4 nadir and HCV coinfection were independently associated with the risk of developing a CVD, apart from other traditional strong predictors of CVD such as lipid levels (total cholesterol and tryglicerides) and diabetes (Tables 2 and 3). Since VZV reactivation and acute bacterial infections nearly reached statistical significance, our aim was to analyze if the cumulative effect of different infections herein considered, acute and chronic, could play a role regarding an increased risk for developing CVD. As shown in Tables 4 and 5, the burden of infections analyzed in the present study was an independent risk factor for CVD in this setting, as well as other well-known risk factors.

4. Discussion

Our manuscript shows that both HCV coinfection and the cumulative effect of different infections increase the risk of developing a CVD in HIV-infected subjects, together with traditional predictor factors of CVD.

Although atherosclerosis is traditionally considered a consequence of lifestyle in developed countries, recent paleogenetic studies have shown that atherosclerosis was present at least 5000 years ago in humans from ancient cultures and in different geographic areas with different lifestyle, diet and genetic backgrounds, reflecting that atherosclerosis development and subsequent CVD is influenced by other conditions together with traditional CVD risk factors (Thompson et al., 2013; Zink et al., 2014). Furthermore, it has been recently reported that if only traditional risk factors are considered, CVD risk prediction may be underestimated mainly in HIV-infected subjects (Long et al., 2018;

Table 2
Factors associated with the development of a CVD. Bivariate regression logistic analysis.

	Univariate		
	OR	IC 95%	*p*
Age at diagnosis, years	1.04	[1.01–1.06]	**0.002**
Male sex	1.49	[0.69–3.2]	0.31
AIDS	2.79	[1.62–4.8]	< **0.001**
T-CD4 nadir < 200 cell/μL	2.69	[1.52–4.8]	**0.001**
IDU[a]	2.24	[1.3–3.84]	**0.003**
HIV diagnosis precART[b]	2.25	[1.29–3.92]	**0.004**
HCV infection[c]	2.32	[1.36–3.98]	**0.002**
Herpes zoster reactivation	2.03	[1.04–3.99]	**0.039**
Acute bacterial infection	1.84	[0.99–3.41]	0.054
Diabetes	4.61	[2.21–9.6]	< **0.001**
Total cholesterol, mg/dl	1.01	[1.005–1.016]	< **0.001**
Triglycerides, mg/dl	1.005	[1.003–1.007]	< **0.001**

[a] Intravenous drug users.
[b] Diagnosis of HIV before 1996, when combined antiretroviral therapy was not available.
[c] HCV: positive HCV-PCR at any time during the observation period.

Table 3
Factors associated with the development of a CVD. Multivariate regression logistic analysis.

	Multivariate		
	OR	IC 95%	*p*
Age at diagnosis, years	1.08	[1.04–1.11]	< **0.001**
AIDS	1.65	[0.89–3.06]	0.11
T-CD4 nadir < 200 cell/μL	2.18	[1.13–4.2]	**0.02**
IDU[a]	1.23	[0.46–3.25]	0.68
HIV diagnosis precART[b]	2.14	[0.99–4.58]	0.05
HCV infection[c]	2.84	[1.1–7.4]	**0.031**
Herpes zoster reactivation, n (%)	1.82	[0.88–3.8]	0.11
Acute bacterial infections, n (%)	1.22	[0.61–2.42]	0.57
Diabetes	3.01	[1.32–6.86]	**0.009**
Total cholesterol, mg/dl	1.011	[1.005–1.02]	< **0.001**
Triglycerides, mg/dl	1.003	[1.001–1.005]	**0.022**

[a] Intravenous drug users.
[b] Diagnosis of HIV before 1996, when combined antiretroviral therapy was not available.
[c] HCV: positive HCV-PCR at any time during the observation period.

Table 4
Factors associated with the development of a CVD, considering the burden of infection. Univariate regression logistic analysis.

	Unadjusted model		
	OR	IC 95%	*p*
Age at diagnosis, years	1.04	[1.01–1.06]	**0.002**
AIDS	2.79	[1.62–4.8]	< **0.001**
T-CD4 nadir < 200 cell/μL	2.69	[1.52–4.8]	**0.001**
IDU[a]	2.24	[1.3–3.84]	**0.003**
HIV diagnosis precART[b]	2.25	[1.29–3.92]	**0.004**
Diabetes	4.61	[2.21–9.6]	< **0.001**
Total cholesterol, mg/dl	1.01	[1.005–1.016]	< **0.001**
Triglycerides, mg/dl	1.005	[1.003–1.007]	< **0.001**
Cumulative infections			
0 (Ref)	1	NA	**0.003**
1	2.09	[1.1–3.99]	**0.025**
≻ 2	3.37	[1.65–6.88]	**0.001**

[a] Intravenous drug users.
[b] Diagnosis of HIV before 1996, when combined antiretroviral therapy was not available.

Table 5
Factors associated with the development of a CVD, considering the burden of infection. Multivariate regression logistic analysis.

	Adjusted model		
	OR	IC 95%	*p*
Age at diagnosis, years	1.07	[1.03–1.01]	< **0.001**
AIDS	1.75	[0.95–3.24]	0.074
T-CD4 nadir < 200 cell/μL	2.01	[1.04–3.85]	**0.037**
IDU[a]	1.67	[0.72–3.87]	0.24
HIV diagnosis precART[b]	2.35	[1.12–4.94]	**0.025**
Diabetes	2.95	[1.31–6.68]	**0.009**
Total cholesterol, mg/dl	1.01	[1.004–1.016]	**0.001**
Triglycerides, mg/dl	1.003	[1.001–1.005]	**0.021**
Cumulative infections			
0 (Ref)	1	NA	
1	2.05	[0.95–4.42]	0.067
≻ 2	3.63	[1.48–8.9]	**0.005**

[a] Intravenous drug users.
[b] Diagnosis of HIV before 1996, when combined antiretroviral therapy was not available.

Triant et al., 2018), concordant with data presented herein in which CVD is developed in HIV-infected subjects with low cardiovascular risk attending to traditional risk factors.

Although HIV-1 infection is a well-known CVD risk factor (Freiberg et al., 2013; Nou et al., 2016), our results suggest that HCV has a cumulative effect in HIV-infected subjects, increasing the risk of developing CVD. The role of HCV as a CVD risk factor has been discussed and discordant data have been reported (Arcari et al., 2006; Oliveira et al., 2013; Wong et al., 2014). However, in the context of HIV infection, HCV-coinfection seems to have major importance regarding the risk of CVD as we show in our work and recently described by other groups (Fernádez-Montero et al., 2016). Additionally, a low T-CD4 nadir showed to be an independent predictor of CVD in HIV-1 infected subjects. Our results clearly suggest that CVD could be ameliorated in HIV-1 infected subjects through two strategies: 1) starting cART as soon as possible, in order to avoid HIV-1 progression and low T-CD4 nadir; 2) treatment of HCV-coinfection with direct antiviral drugs, in order to reduce chronic systemic inflammation secondary to HCV replication.

Regarding VZV reactivation, recent data have shown an association with the risk of developing a cardiovascular event among HIV-1 uninfected individuals (Mnassian et al., 2015). According to our results, VZV reactivation itself showed no independent association with the risk of CVD in HIV-1 infected subjects. However, the cumulative effect of different acute bacterial infections, VZV reactivation and HCV infection increased the cardiovascular risk in the context of HIV-1 infection.

As mentioned above, HIV-1 itself is a cardiovascular risk factor, but an optimal immunovirological response to cART is not enough to prevent CVD, since it has been shown that low grade systemic inflammation and endothelial dysfunction biomarkers remain at high levels after successful cART (Beltrán et al., 2014; Méndez-Lagares et al., 2013). Hence, apart from an effective cART and control of classical predictor factors of CVD risk, immunization for different infections (VZV, pneumococco) and high effective-well tolerated therapy for chronic HCV infection must be considered. The potential role of these strategies for preventing CVD in HIV-1 infected subjects should be addressed in further studies.

Underlying mechanisms through which CVD is increased in HIV-1 infected patients may be similar to those observed in chronic HCV infection and cumulative infections, such as immune hyperactivation and low-grade systemic inflammation driving to endothelial dysfunction (Nou et al., 2016; Peters et al., 2014). In this setting, we have shown that HIV-1 infected subjects with the toll-like receptor 4 Asp299Gly polymorphism cause high levels of proinflammatory cytokines (Tarancón-Díez et al., 2018), reflecting the role of innate immunity in the development of CVD (Fuster, 2018). Therefore, different bacteria in the atheroma plaque of patients with both asymptomatic atherosclerosis and after acute coronary syndrome have been found (Pessi et al., 2013; Rosenfeld et al., 2011). Moreover, an association between infectious diseases and the further development of ischemic events has been described in the last decade (Burgner et al., 2015; Qanitha et al., 2016; Smeeth et al., 2004).

Our study has several limitations, such as the retrospective design. Additionally, pre-cART era was also considered when mortality was mainly due to AIDS-related conditions and CVD was scarcely represented. Another limitation is the burden of infections considered for the present study. It could be discussed if other acute and chronic infections should have been included, but we have considered those consistently documented in clinical records and in which an association with CVD has been previously reported in different clinical setting apart from HIV-1 infection. Finally, the use of statins has not been considered, since prescription of this drug rely on the family doctor and was not strictly controlled.

4.1. Conclusions

Results presented herein show that CVD in the context of HIV infection is influenced by other factors apart from traditional CVD risk factors, acquiring a relevant role both HCV coinfection and the burden of infections. According to these results, potential strategies to minimize CVD risk in these subjects could be treating HCV coinfection in all subjects independently the liver fibrosis stage, starting cART as soon as possible and immunization for those infections in which effective vaccine are available.

Declarations of interest

None.

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Association of heterozygous CCR5Δ32 deletion with survival in HIV- infection: A cohort study

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Association of heterozygous CCR5Δ32 deletion with survival in HIV-infection: A cohort study

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ABSTRACT

The role of a 32 base pair deletion in the CCR5 gene (CCR5Δ32) in HIV-disease progression and response to combined antiretroviral therapy (cART) is well established. However, the impact of CCR5Δ32 in the long-term survival pre-cART and after cART introduction in a large cohort of HIV-infected patients is unknown. We analyzed the association of CCR5Δ32 deletion in the long-term survival of HIV-infected patients recruited between June 1981 and October 2016 (n = 1006). Clinical and epidemiological variables were recorded and CCR5Δ32 deletion was assessed by PCR and electrophoretic analysis. The association of CCR5Δ32 deletion with the time to death was analyzed by Log-Rank tests and Cox Regression models. The CCR5 WT/Δ32 prevalence was 13.4% (n = 135). We did not find any homozygous subject for CCR5Δ32 deletion. AIDS (n = 85, 41.5%) and non-AIDS (n = 87, 42.4%) events were the main causes of 205 deaths. CCR5Δ32 deletion was independently associated with survival (p = 0.022; hazard ratio (HR): 0.572, confidence interval (CI) [0.354–0.923]), after adjusting by HIV diagnosis before 1997, age at diagnosis, being on cART, risk of transmission, nadir $CD4^+$ T-cell counts and CDC stage C. This result was reproduced when the analysis was restricted to patients on cART (p = 0.045; HR: 0.530 [0.286–0.985]). These results confirm the protective role of CCR5Δ32, and extend it to the long-term survival in a large cohort of HIV-infected patients. Beyond its antiviral effect, CCR5Δ32 enhanced the long-term survival of patients on cART.

Seminal publications showed that individuals heterozygous for a 32 base pair (bp) deletion in the gene encoding the chemokine receptor and HIV co-receptor, CCR5 (CCR5Δ32) slowly progress to AIDS and show lower all-cause mortality than subjects with the two wild type (WT) alleles for this mutation (Dean et al., 1996, Huang et al., 1996, de Roda Husman et al., 1997, Barroga et al., 2000). In addition, it was shown that homozygous subjects for this deletion are resistant to HIV infection, with the exception of anecdotal cases (Dean et al., 1996; Henrich et al., 2015). However, these studies were carried out when combined antiretroviral treatment (cART) was still unavailable. Data from patients on cART well demonstrate that CCR5Δ32 is associated with the successful response to antiretroviral treatment regarding $CD4^+$ T-cell levels and HIV-RNA suppression (Valdez et al., 1999), while an association of CCR5Δ32 with mortality on cART has not been found (Brumme et al., 2005; Parczewski et al., 2011).

Therefore, the aim of the present study was to analyze the influence of the CCR5Δ32 deletion on the long-term mortality in a large cohort of HIV-infected patients covering up to of 35 years of follow up, including patients on cART.

Since June 1981 to October 2016, 1006 out of 1062 subjects belonging to the HIV-infected patients' seroprevalence cohort seen at the Virgen del Rocio University Hospital and who had available DNA samples at Immunovirology Laboratory/Biomedicine Institute of Seville (IBiS) were included in the present study. Immunological, virological and clinical data, including non-AIDS events, as previously reported (Pacheco et al., 2015; Dominguez-Molina et al., 2016), were routinely recorded for all the patients included in this study.

CCR5Δ32 deletion was assayed as previously described (Vallejo

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et al., 2006). Additional laboratory measurements can be found in the Supplementary Material.

The outcome variable of the present study was all-cause mortality defined as death by AIDS, non AIDS events, accidental or unknown causes. The target variable was the presence of CCR5Δ32 deletion, while other analyzed covariates were: sex, age at diagnosis, hepatitis C virus (HCV) coinfection, HIV diagnosis before 1997 representing the date when the cART became available, risk category, receiving cART at some point during the follow up, nadir $CD4^+$ T-cell count and Centers for Diseases Control (CDC) category C event at diagnosis. Individuals were observed from HIV diagnosis or cART start date until the date of the event, moment at loss to follow-up or until the study was censored in October 2016 for the remaining subjects. Log rank test and Kaplan-Meier curves were used for time to event analyses, variables with a p value < 0.1 were included in a multivariate analysis consisting in a Cox regression model, using the enter procedure. A linear regression analysis was applied to determine variables independently associated with $CD4^+$ T-cell levels. All variables showing a p value < 0.05 were considered statistically significant. IBM SPSS Statistics software 22.0 version was used for statistical analysis. Graphs were generated using GraphPad Prism, version 6.0 (GraphPad Software, Inc.).

The median [interquartile range, IQR] follow up was 14 [7–23] years and the maximum follow up was 35 years. The median [IQR] age at diagnosis was 30 [24–37] years, 80.2% of the patients were men, 41.8% of the subjects acquired HIV-infection through intravenous drugs use (IDU) and 87.9% were on cART at some point during the follow up (Table 1). The frequency of CCR5Δ32 deletion in heterozygosis (CCR5 WT/Δ32) was 13.4% (n = 135), similar to previously published data in Caucasians (Galvani and Slatkin, 2003). No homozygous subject for CCR5Δ32 deletion was identified.

The total number of deaths during the observation period were 205 (20.4%); 85 (41.5%) and 87 (42.4%) were due to AIDS and non-AIDS events, respectively, the remaining 33 deaths (16.1%) for unknown or accidental causes. A time to event analysis revealed that being diagnosed before 1997, older age at diagnosis, IDU as risk of transmission, having a lower nadir $CD4^+$ T-cell count and showing a CDC stage C during the follow up, were risk factors associated with all-cause mortality (Table 2). On the other hand, being on cART at some time point and carrying CCR5 WT/Δ32 were protective factors associated with survival (Table 2). CCR5 WT/Δ32 remained independently associated with survival after adjusting by all covariates analyzed herein, all of which were strongly associated with time to death. CCR5 WT/Δ32 subjects had an almost two-fold probability to survive during the follow-up as compared to heterozygous subjects, after the median of 14 years of follow up, 13.3% (n = 116) of CCR5 WT/WT subjects died versus 8.1% (n = 11) of CCR5 WT/Δ32 subjects (Table 2).

An additional potential confounding factor in this association is HCV coinfection. In an analysis conducted in the 932 patients who had information on the HCV coinfection status adjusted by HCV exposure, as well as the covariates mentioned above (risk category was not included because it was a co-lineal variable with HCV co-infection), CCR5 WT/Δ32 was independently associated with all-cause mortality (Supplementary Table 1).

Accidental or unknown deaths may bias our observations. When we restricted the analysis to deaths caused by AIDS and non AIDS events as a composite variable, an independent association with CCR5 WT/Δ32 with two fold probability of survival after adjusting by covariates was observed (Supplementary Table 2). Kaplan-Meier curves show the probabilities to achieve the event along time for the different variables independently associated to death (Fig. 1).

In order to demonstrate that CCR5 WT/Δ32 was associated with survival after cART introduction, only patients on cART at any point during the follow-up were selected. In this subpopulation, the cART start date was selected as the analysis initiation time point and plasma viral load at cART initiation was included as a covariate. After adjusting by this variable and again by diagnosis before 1997, age at diagnosis, risk of transmission, $CD4^+$ T-cell nadir and CDC C category, CCR5 WT/Δ32 remained independently associated with time to death: after a median of 9.8 years of follow up, 11.1% (n = 83) of CCR5 WT/WT subjects died compared to 2.6% (n = 3) of CCR5 WT/Δ32 subjects (Table 3).

In addition to patients on cART the relationship of CCR5 WT/Δ32 with $CD4^+$ T-cell counts and viral load was assessed. Interestingly, CCR5 WT/Δ32 subjects had higher $CD4^+$ T-cell levels at the moment starting cART compared to CCR5 WT/WT subjects (345 [204–474] cells/mm^3 vs 273 [127–423] cells/mm^3, p = 0.005). This difference remained after adjusting by viral load at starting cART, age at diagnosis, CDC C category, diagnosis before 1997 and risk of transmission (Supplementary Table 3). Although $CD4^+$ T-cell counts were associated with CCR5 WT/Δ32, viral load levels before cART were not associated with CCR5 WT/Δ32 (p = 0.263).

The present study demonstrates that CCR5Δ32 deletion in heterozygosis is independently associated with improved survival of HIV-infected patients after long-term follow up in a large cohort disregarding the administration of cART.

The long-term follow up of this cohort with 25.0% of the subjects having more than 22 years of follow up, together with the large number of patients, may explain the discrepancies between results presented herein and previous reports that did not find an association between CCR5Δ32 and survival in patients on cART (Brumme et al., 2005; Parczewski et al., 2011). Brumme et al. who found no association (Brumme et al., 2005), analyzed only five years of follow up of naïve HIV-infected patients initiating cART in spite of a similar-sized cohort (n = 1174). Likewise, Parczewski et al. (Parczewski et al., 2011) analyzed factors associated with cumulative 15 years all-cause mortality but despite the relative long-term follow up, no association of CCR5Δ32 deletion with improved survival in the subgroup of patients on cART was observed, probably because only half of the number of patients were included compared to the present study and the observation period was different, only between 1996 and 2010.

Apart from traditional factors associated with all-cause mortality in HIV-infected patients as age, nadir $CD4^+$ T-cell levels, AIDS and antiretroviral treatment (Palella et al., 1998; Miller et al., 1999; Edwards et al., 2015) we found an association with IDU as risk factor. In our geographical area this route of transmission is associated with high prevalence of HCV co-infection. When restricting the analysis to the subgroup of patients with HCV-exposition data available, CCR5Δ32 deletion was still associated with improved survival after adjusting by HCV co-infection. HCV-coinfection was associated with mortality, in accordance with previous studies (Klein et al., 2016; Alejos et al., 2016). The strength of the association of CCR5 WT/Δ32 genotype with mortality was also found when a sensibility analysis restricted to deaths by AIDS and non AIDS events was performed, confirming the independent association of CCR5 WT/Δ32 genotype with all-cause

Table 1
Characteristics of the study subjects.

Characteristic	Value
Follow up time (years), median [IQR]	14 [7–23]
Male sex, n (%)	807 (80.2)
Age at diagnosis (years), median [IQR]	30 [24–37]
HIV diagnosis before 1997, n (%)	512 (50.9)
IDU[a], n (%)	421 (41.8)
CDC category C[b], n (%)	308 (30.6)
Subjects on cART[c], n (%)	884 (87.9)
$CD4^+$ T-cell nadir (cells/mm^3), median [IQR]	193 [72–325]
CCR5 W/Δ32 subjects, n (%)	135 (13.4)
All cause deaths, n (%)	205 (20.4)

[a] Injecting drug user.
[b] Centers for Disease Control and prevention.
[c] Combination antiretroviral therapy at any time during the follow up. The combination of antiretrovirals were chosen based in consensus guidelines along the time.

Table 2
Factors associated with time to death.

	Unadjusted *P* value; HR [95% CI]	Adjusted *P* value; HR [95% CI]
Male sex	0.452; 1.151 [0.799–1.658]	
Age at HIV diagnosis ≥30 years old[a]	**< 0.001; 2.029 [1.518–2.713]**	**< 0.001; 2.033 [1.482–2.791]**
Nadir CD4 T-cells/mm^3	**< 0.001; 0.995 [0.993–0.996]**	**< 0.001; 0.996 [0.994–0.997]**
CDC category C[b]	**< 0.001; 3.984 [2.980–5.326]**	**< 0.001; 2.163 [1.543–3.033]**
IDU[c]	**< 0.001; 1.801 [1.325–2.470]**	**0.005; 1.578 [1.145–2.173]**
Subjects on cART[d]	**< 0.001; 0.123 [0.091–0.168]**	**< 0.001; 0.088 [0.060–0.128]**
HIV diagnosis before 1997	**0.017; 1.548 [1.082–2.214]**	0.052; 1.516 [0.966–2.307]
CCR5 WT/Δ32 subjects	**0.055; 0.629 [0.392–1.010]**	**0.022; 0.572 [0.354–0.923]**

Cox Regression Analysis of factors associated with all-cause mortality. The number of subjects analyzed was 984, there were 199 deaths along 14 [8–23] years of follow-up; HR = hazard ratio; 95% CI = 95% confidence interval.
Variables with a *p* value < 0.1 in the unadjusted model were included in the adjusted model. Variables with a *p* value < 0.05 in the adjusted model were considered statistically significant. Variables with p < 0.1 and p < 0.05 in the unadjusted and adjusted model, respectively, are shown in bold.

[a] The cut off of 30 years old was chosen based on the median age at diagnosis of the cohort.
[b] Centers for Disease Control and prevention.
[c] Injecting drug user.
[d] Combination antiretroviral therapy at any time during the follow up. The combination of antiretrovirals were chosen based in consensus guidelines along the time.

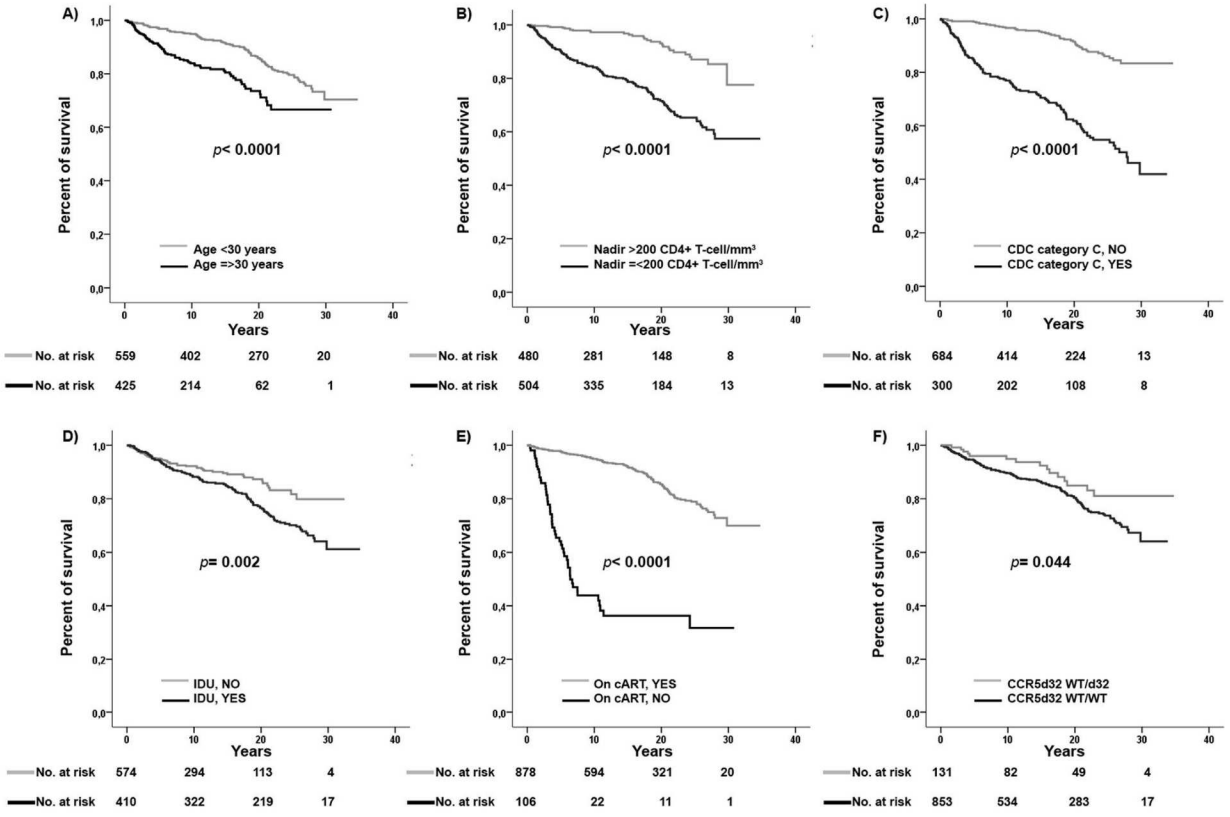

Fig. 1. Kaplan–Meier curves for all time-to death analysis. Percent of survival are shown for (A) Age dichotomized using the median (30 [24–37]) as the cut-off value, (B) Nadir CD4$^+$ T-cell counts using 200 cell/mm^3 as the cut-off value, (C) CDC (Centers for Disease Control and Prevention) category C, (D) Injecting Drug User (IDU), (E) being on cART at any time during the follow up and (F) CCR5/Δ32 genotype. Bivariate analysis using a Log-rank test was performed to assess significant differences among the curves. Number of subjects at risk (No. at risk) are shown along the follow up.

mortality.

The involvement of CCR5Δ32 deletion in the improved survival in patients on cART potentially has important clinical implications. In this context, maraviroc, a CCR5 antagonist, was in part developed because of the identification of CCR5Δ32 as an important factor in delayed HIV-disease progression. It remains unknown whether the use of cART-containing maraviroc would have similar functional consequences as CCR5Δ32 or even an additive effect of this drug in the long-term survival of CCR5Δ32 subjects. Future studies are needed to address this issue.

The mechanisms through which CCR5Δ32 deletion may influence the survival of HIV-infected patients is unknown. One potential explanation is derived by the assumption that individuals with CCR5Δ32 deletion have lower CCR5 expression levels and, as a consequence, lower HIV replication and HIV reservoir size, that may influence a more preserved immunity. However, conflicting results make this hypothesis

Table 3
Factors associated with time to death in subjects on cART.

	Unadjusted *P* value; HR [95% CI]	Adjusted *P* value; HR [95% CI]
Male sex	0.325; 1.258 [0.796–1.988]	
Age at HIV diagnosis ≥30 years old[a]	**0.063; 1.383 [0.983–1.947]**	**0.001; 1.903 [1.290–2.806]**
Nadir CD4 T-cells/mm^3	**< 0.001; 0.995 [0.994–0.997]**	**< 0.001; 0.997 [0.995–0.998]**
Log HIV-RNA[b]	0.524; 1.059 [0.887–1.265)]	
CDC category C[c]	**< 0.001; 3.282 [2.325–4.632]**	**< 0.001; 2.026 [1.395–2.943]**
IDU[d]	**< 0.001; 2.642 [1.825–3.824]**	**< 0.001; 2.429 [1.628–3.625]**
HIV diagnosis before 1997	**0.036; 1.496 [1.027–2.178]**	0.381; 1.206 [0.793–1.835]
CCR5 W/Δ32 subjects	**0.072; 0.567 [0.306–1.051]**	**0.045; 0.530 [0.286–0.985]**

Cox Regression Analysis of factors associated with all-cause mortality. The number of subjects analyzed was 864, there were 133 deaths along 10 [6–18] years of follow-up since the onset of cART; HR = hazard ratio; 95% CI = 95% confidence interval.
Variables with a p value < 0.1 in the unadjusted model were included in the adjusted model. Variables with a p value < 0.05 in the adjusted model were considered statistically significant. Variables with $p < 0.1$ and $p < 0.05$ in the unadjusted and adjusted model, respectively, are shown in bold.

[a] The cut off of 30 years old was chosen based on the median age at diagnosis of the cohort.
[b] Viral load expressed as Log_{10} HIV-RNA copies/mL. This variable was available in 698 patients.
[c] Centers for Disease Control and prevention.
[d] Injecting drug user.

uncertain at the moment (Wang et al., 2014; Henrich et al., 2016). Unknown mechanism/s may account to a more complex explanation than CCR5Δ32 deletion equals to lower CCR5 expression. In fact, CCR5 expression can widely vary in wild type and CCR5Δ32 heterozygous subjects with the possibility of both genotypes harboring the same CCR5 expression levels (Catano et al., 2011). This is because, importantly, other polymorphisms close to the CCR5Δ32 deletion region and the CCR5 promoter region (haplotype P1), highly impact on CCR5 expression and may refer to functional variants with unknown functions (McLaren et al., 2015). Some authors have assigned this finding, at least in part, to increased T-cell immunity, associated with the presence of particular CCR5 polymorphisms and haplotype pairs, as the responsible of diminished HIV progression associated with CCR5Δ32 heterozygosis (Catano et al., 2011).

This study has limitations. First, all the patients had non-AIDS events data recorded, however we did not have enough statistical power to analyze the influence of CCR5Δ32 deletion in deaths caused by this type of events. Second, our study was restricted to CCR5Δ32 deletion, when other genetic variants or epigenetic modifications in the CCR5 genome region may be associated with mortality in HIV-infection. Additional studies are warranted to clarify this matter. Third, we could not include viral load in the analysis of the whole cohort (Table 2) since this variable is time-dependent and determinations were unavailable in 30.0% of the subjects, most of whom were diagnosed before 1997 when viral load assays were not available. However, when we included viral load as a covariate in the model of subjects on cART, CCR5Δ32 deletion was still independently associated with survival.

In summary, this study confirms and extends the role of CCR5Δ32 as a predictive factor for survival in HIV-infection. Particularly important is that beyond its antiviral effect, CCR5Δ32 enhances the long-term survival also of those patients receiving cART.

Declaration of interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2017.12.002.

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Factors leading to the loss of natural elite control of HIV-1 infection

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PATHOGENESIS AND IMMUNITY

Factors Leading to the Loss of Natural Elite Control of HIV-1 Infection

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ABSTRACT HIV-1 elite controllers (EC) maintain undetectable viral loads (VL) in the absence of antiretroviral treatment. However, these subjects have heterogeneous clinical outcomes, including a proportion that loses HIV-1 control over time. In this work, we compared, in a longitudinal design, transient EC, analyzed before and after the loss of virological control, with persistent EC. The aim was to identify factors leading to the loss of natural virological control of HIV-1 infection with a longitudinal retrospective study design. Gag-specific T-cell responses were assessed by *in vitro* intracellular polycytokine production quantified by flow cytometry. Viral diversity determinations and sequence dating were performed in proviral DNA by PCR amplification at limiting dilution of *env* and *gag* genes. The expression profile of 70 serum cytokines and chemokines was assessed by multiplex immunoassays. We identified transient EC as subjects with low Gag-specific T-cell polyfunctionality, high viral diversity, and high proinflammatory cytokine levels before the loss of control. Gag-specific T-cell polyfunctionality was inversely associated with viral diversity in transient controllers before the loss of control ($r = -0.8$; $P = 0.02$). RANTES was a potential biomarker of transient control. This study identified virological and immunological factors, including inflammatory biomarkers associated with two different phenotypes within EC. These results may allow a more accurate definition of EC, which could help in better clinical management of these individuals and in the development of future curative approaches.

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IMPORTANCE There is a rare group of HIV-infected patients who have the extraordinary capacity to maintain undetectable viral load levels in the absence of antiretroviral treatment, the so-called HIV-1 elite controllers (EC). However, there is a proportion within these subjects that eventually loses this capability. In this work, we found differences in virological and immune factors, including soluble inflammatory biomarkers, between subjects with persistent control of viral replication and EC that will lose virological control. The identification of these factors could be a key point for a right medical care of those EC who are going to lose natural control of viral replication and for the design of future immunotherapeutic strategies using as a model the natural persistent control of HIV infection.

KEYWORDS HIV-1 elite controllers, T-cell response, viral diversity, inflammatory biomarkers, HIV-1 controllers, inflammation

The spontaneous control of human immunodeficiency virus type 1 (HIV-1) infection is observed in a rare group of subjects known as HIV-1 elite controllers (EC) (1). As these individuals maintain undetectable viral loads (VL) in the absence of antiretroviral treatment, they have been proposed as a model of functional cure (2). The investigation of the mechanisms behind this natural control has attracted enormous interest for the identification of the host and the virological factors implicated in this phenomenon (3–6).

However, some of these individuals experience virological and immunological progression and AIDS and non-AIDS defining events (7–9). Regarding virological progression, approximately 28% of EC experience loss of viral control over time (7). The factors associated with this loss remain elusive due to the different designs of the studies. First, previous studies were limited to cross-sectional analyses in which virological progression and the heterogeneous characteristics of HIV-1 controllers were not widely taken into account (6, 10–13). Second, there have been very few longitudinal studies, mostly epidemiological (14), where the main contributors were higher ultrasensitive HIV-1 RNA VL and proviral DNA levels. Furthermore, nonconclusive results about inflammatory biomarkers have been found (15). Therefore, the specific determinants associated with virological failure in EC are not definitely established.

The objective of this work was to investigate, following a longitudinal study design, the mechanisms leading to the loss of virological control in a cohort of EC. To this end, we carried out an exhaustive analysis of virological and immunological factors, including proinflammatory cytokines, that could explain the transient or persistent nature of virological control in HIV-1 infection. The identification of biomarkers associated with the loss of viral control will allow the identification of this subgroup of EC, which should help to improve their medical care. In addition, the identification of those factors operating in the persistent control of viral replication in EC may provide new insights for the design of novel eradication and immunotherapeutic strategies.

RESULTS

Characteristics of the studied subjects. Clinical and demographic characteristics of transient controllers (TC) and persistent controllers (PC) (see the study design in Fig. 1) are shown in Table 1. The frequency of a sexual transmission route was higher in TC (75%) than in PC (37%) ($P = 0.049$). The TC group presented a shorter time after diagnosis than the PC group (8 [2 to 14] years versus 18 [11 to 22] years; $P = 0.002$). There were no differences in the remaining variables at baseline. After the loss of control, the VL from TC were 627 (230 to 4,618) HIV RNA copies/ml at time zero (T0), 1,730 (397 to 4,420) HIV RNA copies/ml at 1 year (+T1), and 2,860 (727 to 4,920) HIV RNA copies/ml at 2 years (+T2). Therefore, half of the patients were viremic controllers because of the loss of control, and the remaining patients had low VL ($<10^4$ log HIV RNA copies/ml).

Low levels of Gag-specific T-cell responses preceded the loss of virological control in TC. The $CD4^+$ and $CD8^+$ T-cell counts and CD4/CD8 ratios were not different

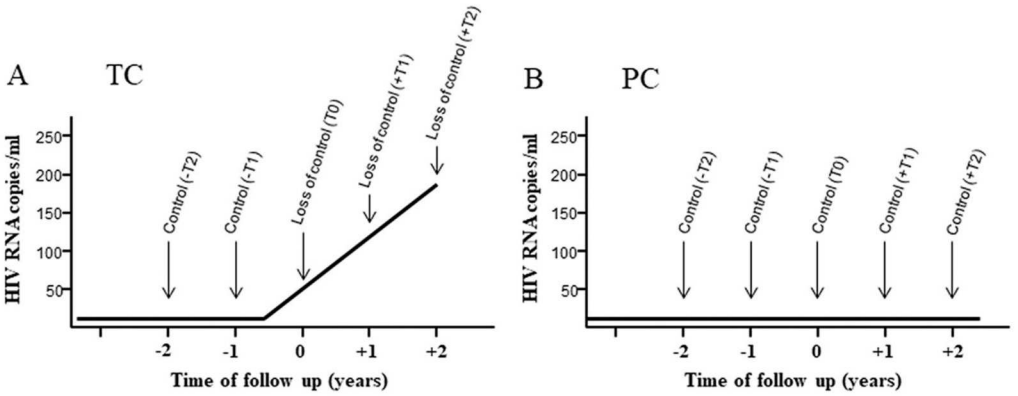


FIG 1 Study design. Schematic representation of the longitudinal and retrospective study design in transient controllers (TC) (A) and persistent controllers (PC) (B). In TC, up to five determinations were performed: two in the "under-control period," 2 years (−T2) and 1 year (−T1) before the loss of control, and up to three determinations in the "post-loss-of-control period," including the closest time point to the loss of virological control (T0) and 1 year (+T1) and 2 years (+T2) after the loss of virological control. At least the −T2, −T1, and T0 samples were required for the subject to be included in the study. In total, a maximum of 54 time points were analyzed in this group. In PC, up to five determinations were performed at 1-year intervals, but at least three consecutive time points per subject were required to be included in the study. In total, a maximum of 63 time points were analyzed in this group. For Gag-specific T-cell response assays, all available follow-up time points were tested (PC, $n = 14$; TC, $n = 14$). Virological and soluble biomarkers assays were done for all available follow-up time points in the PC ($n = 10$ and $n = 11$, respectively) and only at –T2 and –T1 in the TC ($n = 9$ and $n = 12$, respectively).

and did not change during follow-up in the two groups (Table 2). In the same way, no differences were observed throughout the follow-up in Gag-specific $CD4^+$ and $CD8^+$ and different T-cell subset responses in PC after multiple comparison testing (Fig. 2). Based on these data and to simplify the analyses, the variables in PC were expressed as the mean value of all longitudinal determinations.

Importantly, there was a higher proportion of PC (12/14 subjects [85.7%]) who showed Gag-specific $CD4^+$ T-cell responses than of TC (3/13 subjects [23.1%]) at 1 year before the loss of virological control (−T1), when Gag-specific $CD4^+$ T-cell responses dramatically decreased. No statistical differences were observed between PC and TC 2 years before (−T2) the loss of virological control nor at the time point just after the loss of virological control (T0) (Fig. 3A, left panel). No differences were found in $CD8^+$ T-cell

TABLE 1 Characteristics of the subjects

Parameter[a]	Value for group[b]		
	TC ($n = 14$)	PC ($n = 17$)	P^c
Age (yr), median (IQR)	41 (38–52)	45 (41–48)	0.279
Male sex, no. (%)	8 (57)	10 (59)	0.925
Sexual transmission, no. (%)	10 (71)	6 (35)	**0.049**
Time since diagnosis (yr), median (IQR)	8 (2–14)	18 (11–22)	**0.002**
HCV RNA detected, no. (%)	6 (43)	10 (59)	0.376
$CD4^+$ T cells (cells/μl), median (IQR)	625 (392–783)	714 (627–940)	0.208
$CD8^+$ T cells (cells/μl), median (IQR)	735 (548–1015)	648 (569–970)	0.999
CD4/CD8 ratio, median (IQR)	1 (0.58–1.28)	1.17 (0.67–1.55)	0.456
HLA B57, no. (%)[d]	3 (21)	6 (40)	0.280
HLA B27, no. (%)[d]	1 (7)	2 (13)	0.584
HLA B35, no. (%)[d]	2 (14)	0 (0)	0.129
IL28B-CC, no. (%)[e]	3 (38)	5 (42)	0.728

[a]IQR, interquartile range.
[b]Values from transient controllers (TC) are taken from −T2, and values from persistent controllers (PC) are taken from the first time point of follow-up.
[c]The Mann-Whitney U and chi-square tests were used. All P values of <0.05 were considered significant and are highlighted in bold.
[d]$n = 15$ for PC.
[e]$n = 8$ and $n = 12$ for TC and PC, respectively.

TABLE 2 T-cell levels and CD4/CD8 ratio of the study subjects[a]

Group	Time point	CD4 T cells (cells/μl)	CD8 T cells (cells/μl)	CD4/CD8 ratio
TC (n = 14)[b]	−T2	625 (391–783)	735 (547–1,014)	1 (0.58–1.28)
	−T1	745 (491–925)	862 (587–1,223)	0.97 (0.66–1.16)
	T0	591 (474–761)	686 (532–1,134)	0.93 (0.44–1.3)
	+T1	531 (424–695)	750 (646–1,147)	0.73 (0.38–1.07)
	+T2	735 (547–1014)	602 (592–1,501)	0.56 (0.45–1.31)
PC (n = 17)[b]	−T2	714 (627–940)	648 (569–970)	1.17 (0.67–1.55)
	−T1	689 (557–940)	720 (483–917)	1.09 (0.81–1.72)
	T0	550 (419–958)	629 (350–1,030)	1.13 (0.51–1.77)
	+T1	710 (600–961)	672 (584–1,058)	1.43 (0.88–1.68)
	+T2	556 (489–661)	554 (251–764)	1.18 (0.78–2.02)
	PC (mean)	651 (576–989)	725 (591–954)	1.09 (0.62–1.56)
P	−T2 vs PC (mean)	0.284	0.757	0.452
	−T1 vs PC (mean)	0.965	0.292	0.547
	T0 vs PC (mean)	0.174	0.906	0.269
	+T1 vs PC (mean)	0.084	0.393	0.052
	+T2 vs PC (mean)	0.351	0.969	0.225

[a]Values are given as medians (interquartile ranges).
[b]The Friedman test was used to analyze differences during the follow-up in each group (TC and PC); no differences were found ($P > 0.05$) in all the cases. The Mann-Whitney U test was used for between-group comparisons.

responses, although there was a similar trend toward lower responses at –T1 in TC (Fig. 3A, right panel).

By analyzing the magnitude and characteristics of the response, we observed that PC presented higher levels of Gag-specific total, central memory (CM), and effector memory (EM) CD4$^+$ T cells than TC at –T1 (Fig. 3B). The same results were obtained for

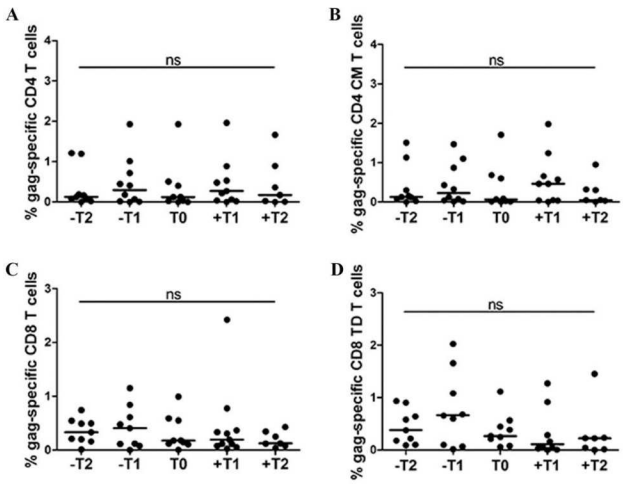


FIG 2 Representative longitudinal Gag-specific T-cell-associated parameters in PC. The T-cell response was defined as the frequency of cells (>0.05% after background subtraction of the unstimulated condition) with detectable IFN-γ, TNF-α, and/or IL-2 intracellular cytokine production after stimulation of cryopreserved PBMCs with Gag overlapped peptides. Gag-specific total CD4$^+$ T-cell response (A), central memory CD4$^+$ T-cell response (B) (CM, CD4 CD45RA CD27), total CD8$^+$ T-cell response (C), and terminally differentiated CD8$^+$ T-cell response (D) (TD, CD8 CD45RA CD27) levels are shown. No statistical differences were obtained throughout the follow-up. NS denotes no significant differences between multiple paired sample comparisons determined by the Wilcoxon signed-rank test ($P > 0.05$ in all cases). The Friedman test could not be applied due to insufficient statistical power using the five follow-up time points.

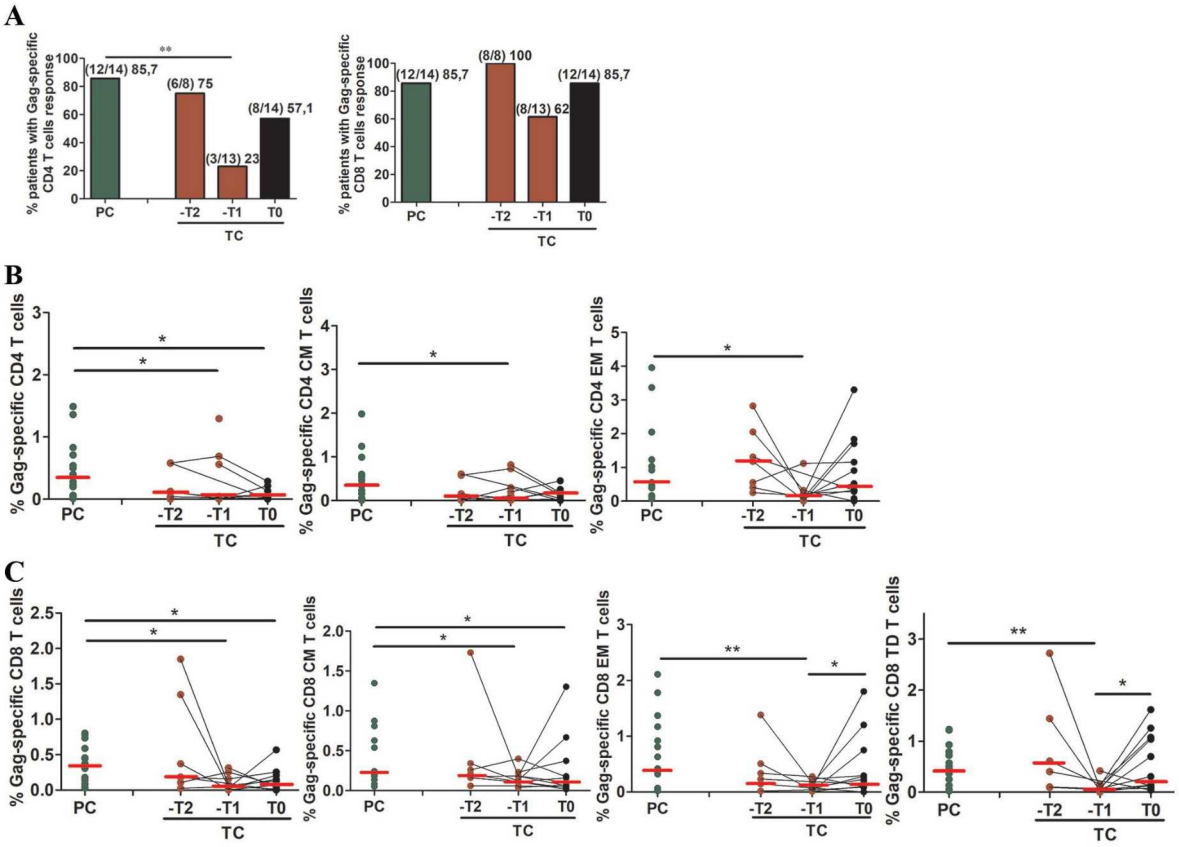


FIG 3 CD4$^+$ and CD8$^+$ T-cell Gag-specific responses. The percentages of subjects with Gag-specific CD4$^+$ and CD8$^+$ T-cell responses are shown. (A) The T-cell response was defined as the frequency of cells (>0.05% after background subtraction of the unstimulated condition) with detectable IFN-γ, TNF-α, and/or IL-2 intracellular cytokine production after stimulation of cryopreserved PBMCs with Gag overlapped peptides. (B) Total, central memory (CM; CD4$^+$ CD45RA$^-$ CD27$^+$), and effector memory (EM; CD4$^+$ CD45RA$^-$ CD27$^-$) Gag-specific CD4$^+$ T-cell levels; (C) total, CM, EM, and terminally differentiated (TD; CD8$^+$ CD45RA$^+$ CD27$^-$) Gag-specific CD8$^+$ T-cell levels. Differences between unpaired groups were determined by the Mann-Whitney U and chi-square tests, and differences between paired samples were determined by the Wilcoxon signed-rank test. The Friedman test was not applied due to the small number of paired samples. Only significant differences are shown. *, $P < 0.05$; **, $P < 0.001$.

CD8 T cells (Fig. 3C). In addition, in TC, increased levels of Gag-specific EM and terminally differentiated (TD) CD8$^+$ T cells were found at T0 compared to –T1 (Fig. 3C).

Gag-specific CD8$^+$ T-cell polyfunctionality is decreased in TC before the loss of virological control. A higher frequency of polyfunctional CD8$^+$ T cells was observed in PC than in TC at –T1, with a higher proportion of cells with three functions and a higher cytokine diversity (a representative example is shown in Fig. 4A). There were no differences between PC and TC at T0, when VL was detectable in TC. However, by analyzing the maturation and activation patterns of Gag-specific T-cell responses (Fig. 4B), we found that TC presented higher Gag-specific CD8$^+$ CD38$^+$ T-cell levels and lower Gag-specific CD8$^+$ CM CD57$^-$ T-cell levels at –T1 and T0 than PC. In addition, bulk T-cell activation was higher in TC than PC at T0 (Fig. 5).

Indexes of polyfunctionality (pINDEX) in Gag-specific total CD8$^+$ T cells were compared between PC and TC at –T2 together with –T1 (pre-loss-of-control period). The pINDEX was higher in three, four, and five functions (Fig. 4C to E) in PC than in TC.

TC displayed higher viral diversity than PC. *env* and *gag* genes were amplified in proviral DNA by limiting-dilution PCR in all TC (9/9, 100%) but in only 5 of 10 (50%) of the PC. Afterwards, in the remaining available samples, we quantified proviral loads by an *Alu* real-time PCR; 33% (4/12) of TC samples showed values ranging from 5 to 108

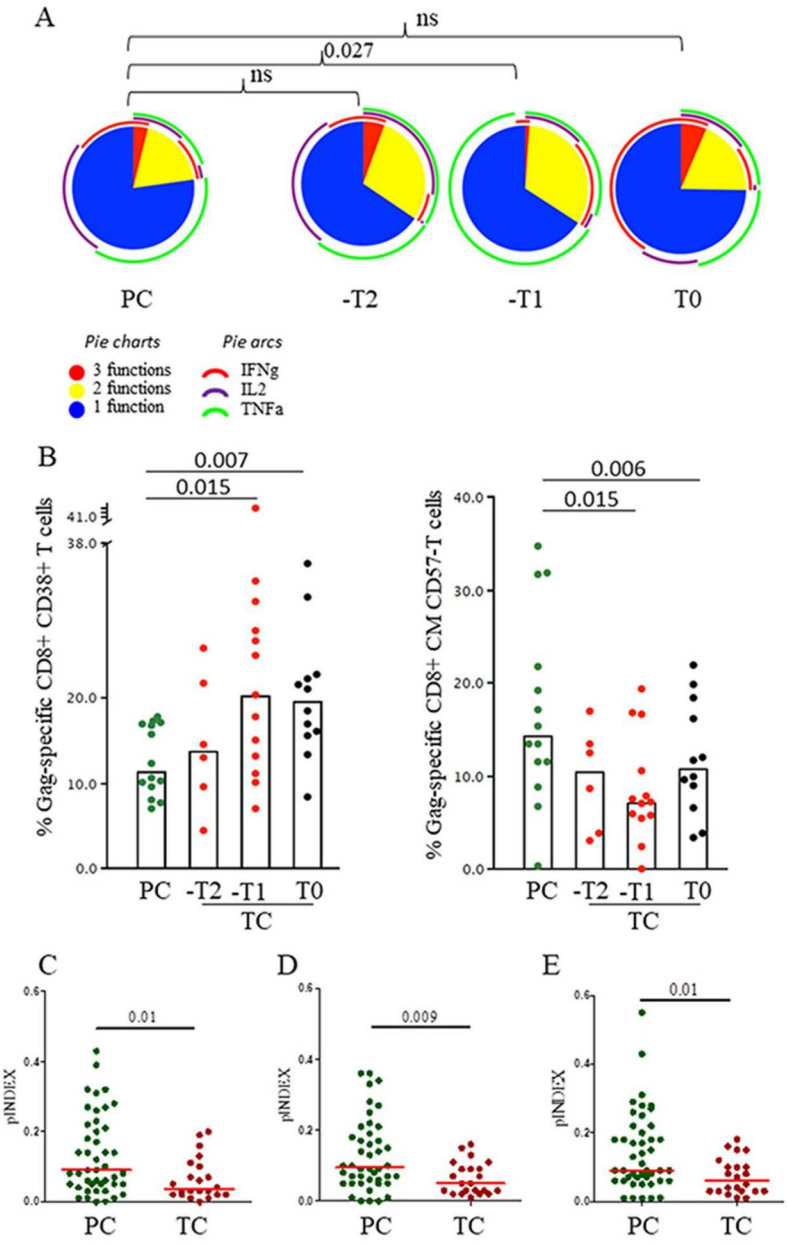


FIG 4 HIV-1-specific CD8$^+$ T-cell polyfunctionality. Polyfunctionality, understood as simultaneous multiple production of IFN-γ, TNF-α, and IL-2 per T cell, was studied only for subjects categorized as responders. The T-cell response was defined as the frequency of cells (>0.05% after background subtraction of the unstimulated condition) with detectable IFN-γ, TNF-α, and/or IL-2 intracellular cytokine production after stimulation. Due to the low number of Gag-specific CD4$^+$ T-cell responders in TC, polyfunctionality analysis was not applicable. (A) Pie charts show polyfunctional distribution of HIV-1-specific CD8$^+$ TD CD57$^+$ T cells with up to three functional responses to Gag stimulation; IFN-γ, TNF-α, and IL-2 production in the polyfunctional distribution is shown in arcs. Pestle and Spice were used for analysis. (B) Percentages of Gag-specific CD8$^+$ T cells expressing the activation profile, CD38$^+$, and the maturation profile, CD45RA$^-$ CD27$^+$ CD57$^-$; only significant differences are shown. (C to E) Polyfunctionality index of Gag-specific total CD8$^+$ T cells. Values from PC and preloss time points of follow-up (–T2 and –T1) in TC are based on the proportions of cells expressing combinations of IFN-γ, TNF-α, and IL-2 (three functions) (C), plus CD107a (four functions) (D), and plus perforin (five functions) (E). Single and double production of CD107a and perforin were excluded from the analyses. Differences between groups were determined by the Mann-Whitney U test.

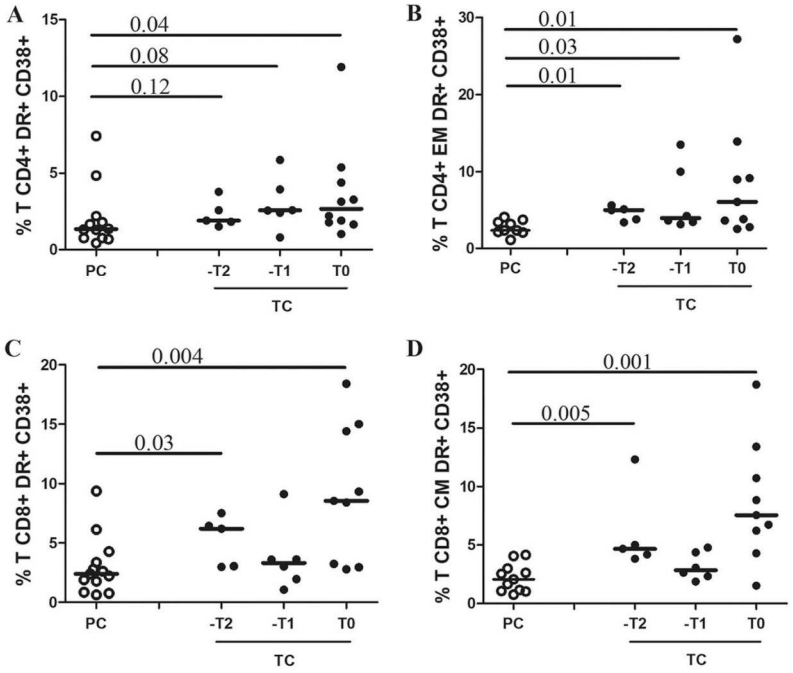


FIG 5 CD4$^+$ and CD8$^+$ T-cell activation. Percentages of total (A) and effector memory (B) CD4$^+$ HLA-DR$^+$ CD38$^+$ T cells and total (C) and central memory (D) CD8$^+$ HLA-DR$^+$ CD38$^+$ T cells are shown. Differences between unpaired groups were determined by the Mann-Whitney U test. The Friedman test was not applied due to the small number of paired samples. $P < 0.15$ are shown.

copies/10^6 cells, and the remaining TC samples and 9 of 9 samples from PC (100%) showed values below the detection limit (5 copies/10^6 cells).

The branches in the phylogenetic tree (Fig. 6A) were longer in TC than in PC, indicating viral replication and evolution. In the sequences from PC, the branch length was minimal or zero, with many identical sequences. Although we did not analyze the integration sites, this pattern indicates a lack of viral replication and could suggest that these viral populations are the consequence of clonal expansions (16–18).

Regarding the evolution of *gag* and *env* gene sequences with time, TC showed intermingled sequences and no replacement of viral populations at the different time points (Fig. 6A). This evolutionary model is compatible with an atemporal mode of evolution (11). In contrast, sequences at different times from PC showed no evidence of viral evolution (Fig. 6A). These results were supported by viral dating estimation. For this analysis, available samples of PC, obtained in 2010 $\pm$ 0.9 year, a mean of 18 years after HIV-1 diagnosis, showed sequences without any viral evolution since HIV-1 diagnosis (Table 3). In these patients, the difference between viral dating and the HIV-1 diagnosis time was 5 years. In TC patients, available samples obtained a mean of 15 years after HIV-1 diagnosis showed, because of viral evolution, a viral dating (2001 $\pm$ 3.4 years) closer to the sampling time (2005 $\pm$ 3.5 years) than to the HIV diagnosis date (1989 $\pm$ 4.1 years), with a mean difference of 11.5 years (Table 3). In two TC (F4 and EC4), the nucleotide sequences were separated in two independent clades (Fig. 6A, right panel). This segregation supports that these were double-infected subjects, as previously reported for EC4 (19). All patients had an R5 tropism, and, in the case of TC, the tropism did not change after viral replication.

Viral diversity in the *gag* region was higher in TC than in PC, but the difference did not reach a statistical significance, probably due to the low number of sequences analyzed. In contrast, in the *env* C2-V5 region, diversity was statistically higher in TC

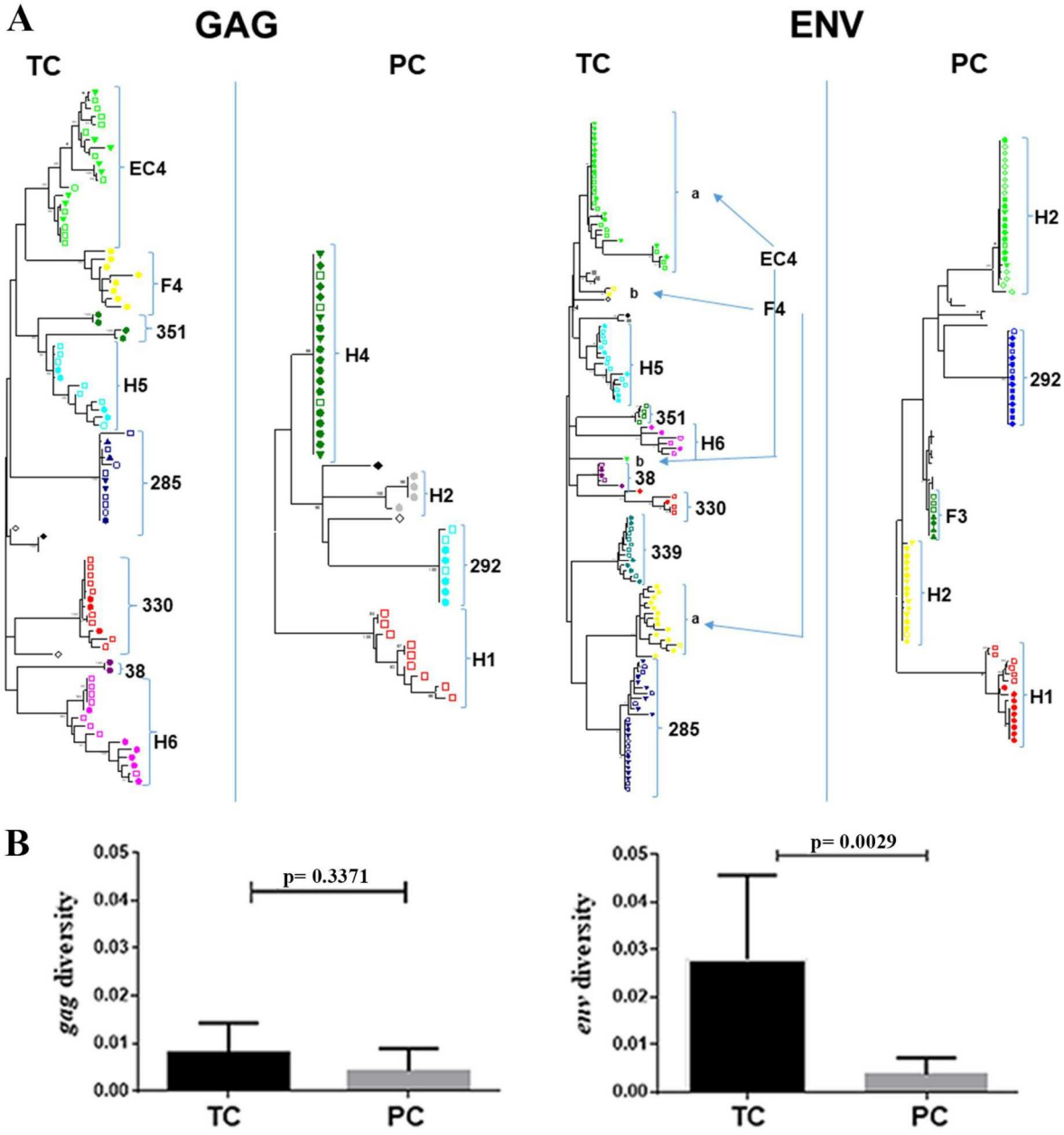


FIG 6 Virological assays: phylogenetic analysis and virus diversity. All virological assays were performed with all time point samples in PC and with only samples from time points prior to the loss of control in TC. Phylogenetic analysis of sequences in *env* and *gag* genes from TC and PC during follow-up was done. Sequences were submitted to GenBank under accession numbers MF988754 to MF989105. (A) Phylogenetic trees were estimated by a maximum likelihood approach using the best-fit model of nucleotide substitution (GTR+G+I; jModelTest v.0.1.1) implemented in the MEGA 6 software program. Each subject is represented by a different color. Samples taken at different times are marked with different symbols. In double-infected subjects (EC4 and F4), the two viral populations are marked and labeled a and b. Bars indicate a genetic distance of 0.02. (B) Comparison between intrasample diversity in *env* and *gag* regions for TC and PC. The mean and standard errors for all pairwise nucleotide distances were determined using the MEGA 6.0 program. Differences between unpaired groups were determined by the Mann-Whitney U test. **, $P < 0.05$.

TABLE 3 Year of HIV-1 diagnosis, sampling year, and viral dating in TC and PC

Patient group and identifier	Yr of HIV-1 diagnosis	Sampling yr	Yrs since HIV-1 diagnosis	Yr estimated by viral dating	Difference in yr of viral dating and HIV-1 diagnosis (yrs)
TC					
330	1991	2000	9	2004	13
F4	1986	2003	17	1997	11.8
74	1996	2007	11	2003.2	7.2
H5	1986	2006	20	1997	11
H6	1990	2009	19	2003.6	13.6
Mean[a]	1989 ± 4.1	2005 ± 3.5	15 ± 4.9	2001 ± 3.4	11.5
PC					
H1	1999	2010	11	2004	5
H2	1994	2011	17	1997	3
H4	1986	2011	25	1993	7
292	1998	2011	13	1995	−3
F3	1985	2009	24	1994	9
Mean[a]	1992 ± 6.6	2010 ± 0.9	18 ± 6.3	1997 ± 4.4	5

[a]Values are expressed as means ± standard deviations. To perform the viral dating of the subjects, the genetic distance of the reconstructed most recent common ancestor (MRCA) for each patient clade was compared with that of a reconstructed MRCA for the Spanish epidemic. The viral dating time was estimated by use of a linear-correlation equation, previously developed in a large set of Spanish samples, that correlates the V3 nucleotide sequence divergence to the Spanish epidemic MRCA and the sampling year. Only four subjects in the TC group were included. The viral dating of other TC could not be estimated because the sampling time and the year of HIV-1 diagnosis were too close in time.

than in PC ($P < 0.0029$), again supporting viral replication in TC (Fig. 6B), even if we exclude analysis of the sequences of the double-infected patients ($P < 0.016$).

Virus diversity was associated with Gag-specific CD8$^+$ T-cell polyfunctionality. Three-function total CD8$^+$ T-cell polyfunctionality was inversely associated with *gag* diversity ($r = -0.8$; $P = 0.02$) but not with *env* (Fig. 7A) in TC. Regarding four and five functions, total CD8$^+$ T-cell polyfunctionality association with viral diversity remained in the same trend as that of three functions, although it was not statistically significant ($r = -0.6$, $P = 0.14$, and $r = -0.57$, $P = 0.1$, for four and five functions, respectively). No association was found in PC (data not shown). We evaluated the presence of HIV-1 footprints of immune escape in Gag epitopes in both pre- and post-loss-of-control samples comparing TC and PC. We found a trend toward a higher frequency of mutations in Gag epitopes in TC than in PC (Fig. 7B). In addition, for TC patient 351 expressing HLA-B*57, we tracked the temporal emergence of escape variants in the HLA-B*57 restricted epitope ISW9, which appeared after the loss of control, and the preexistence of TW10 escape mutants along with the loss of virological control over time, indicating HIV-1 evolution against CD8$^+$ T-cell responses (Fig. 7C).

High soluble biomarkers and proinflammatory cytokine levels preceded the loss of virological control. The higher levels of RANTES, interferon alpha 2 (IFN-α2), human growth-regulated oncogene (GRO), interleukin 7 (IL-7), and cutaneous T-cell-attracting chemokine (CTACK) in TC than in PC were the variables that significantly differentiated the two groups by the Mann-Whitney U test (Fig. 8A). After random forest analysis (Fig. 8B), RANTES, IFN-α2, and CTACK were similarly considered potential biomarkers together with platelet-derived growth factor AA (PDGF-AA) and eotaxin-2. The principal-component analysis (PCA) model (Fig. 8C) restricted the biomarkers to RANTES and PDFG-AA, which showed the best percentage of separation between TC and PC. Finally, analysis by receiver operating characteristic (ROC) curves (Fig. 8D) showed that the top variables of the random forest approach (model B) and the PCA (model C) displayed an area under the curve (AUC) of 1, which means that a perfect classification was achieved using only RANTES and PDGF-AA. All statistical tests defined RANTES as the most remarkable chemokine for group discrimination, with 4-fold higher levels in TC than in PC ($P = 0.001$) and a cutoff value of 10,464.5 pg/ml.

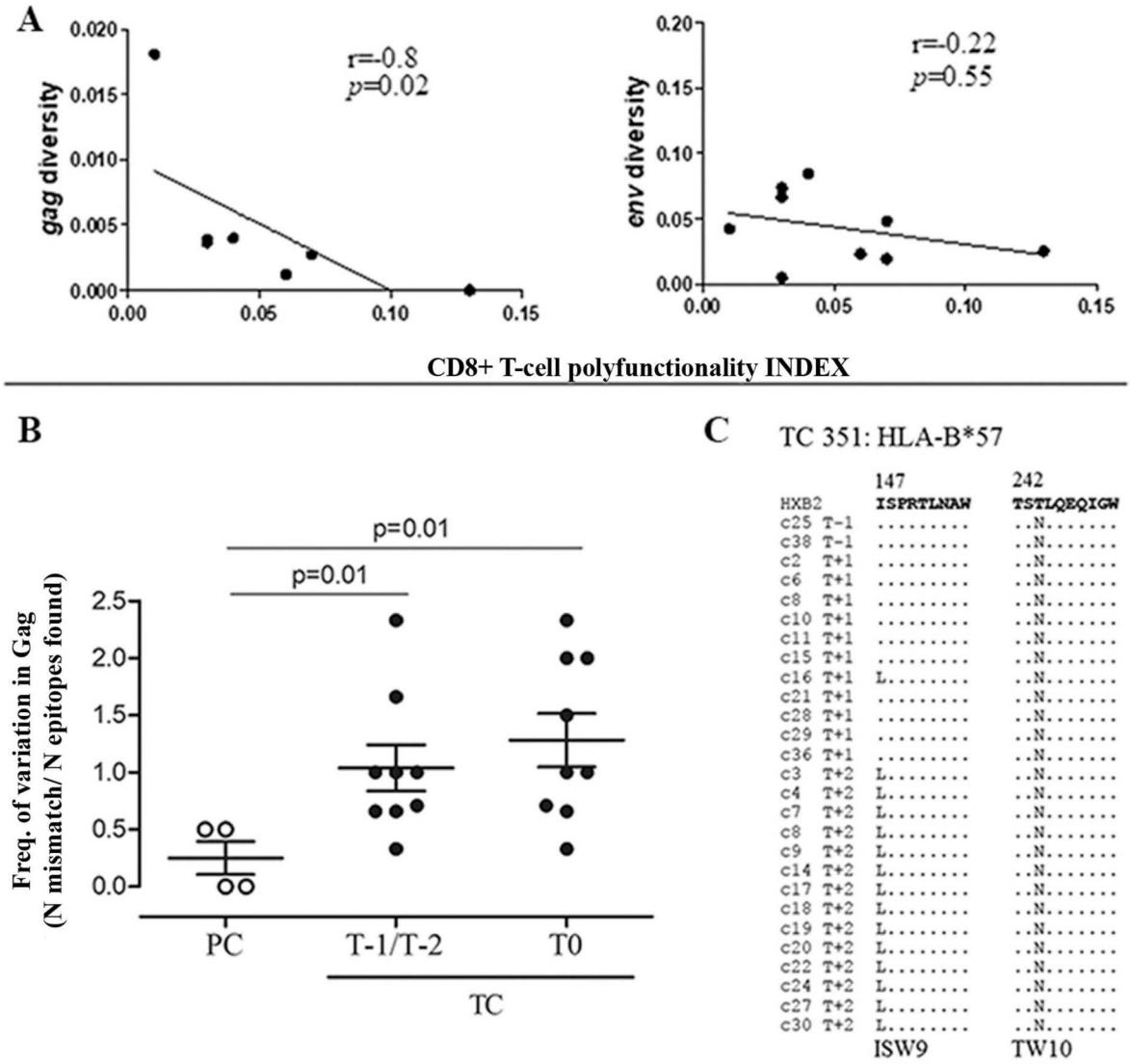
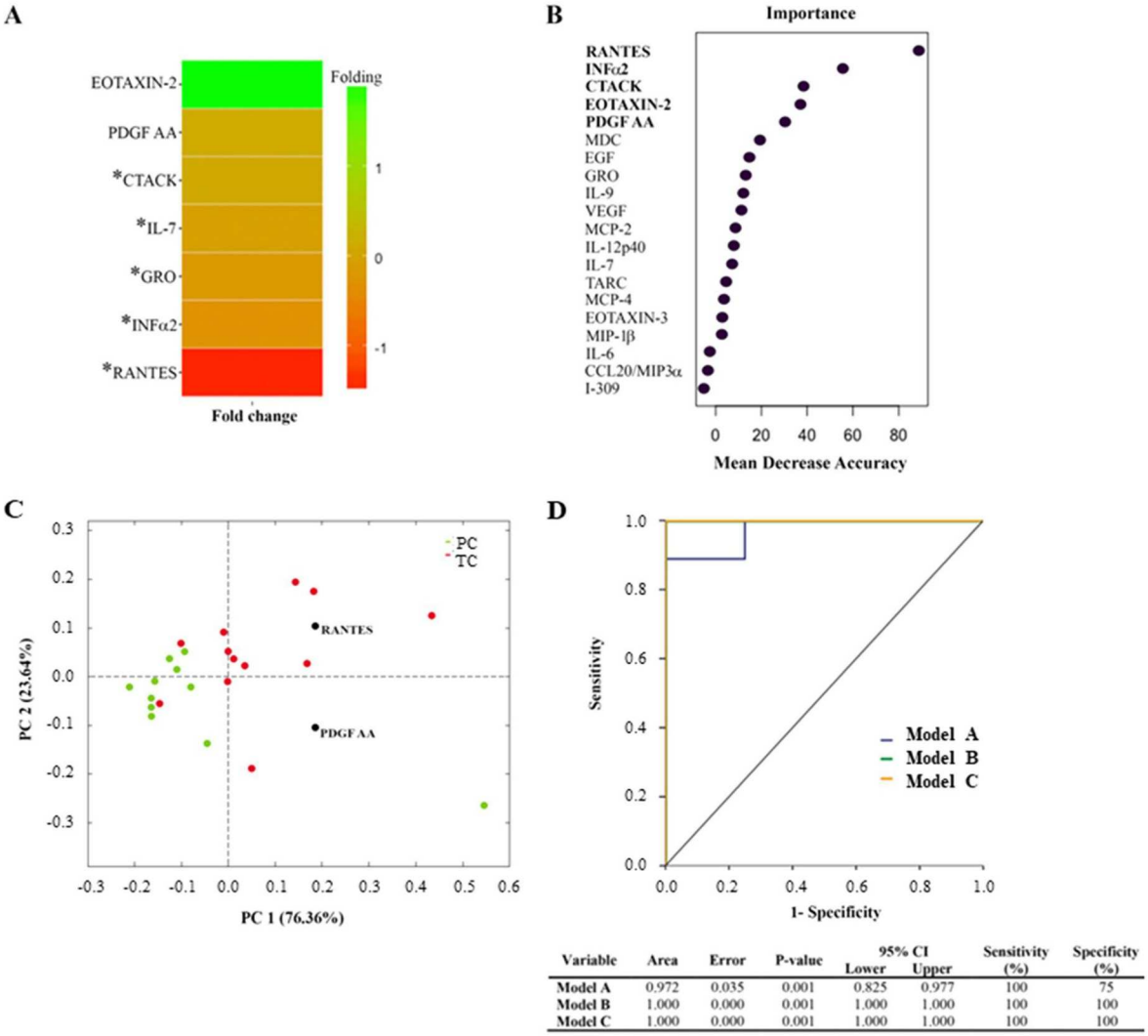


FIG 7 Correlation of virus diversity with CD8$^+$ T-cell polyfunctionality and analysis of Gag CD8$^+$ T-cell epitope variation. (A) Correlations between *gag* and *env* diversity and the total CD8$^+$ T-cell three-cytokine polyfunctionality index (pINDEX for three functions) in TC are shown. The Spearman rho correlation coefficient test was used. (B) Analysis of HIV-1 Gag CD8$^+$ T-cell epitope variation. The frequencies (Freq.) of variation in HIV-1 Gag CD8$^+$ T-cell epitopes in PC and TC before and after the loss of virological control are shown. Differences between groups were determined by the Mann-Whitney U test. (C) HIV-1 sequence variation in Gag ISW9 and TW10 epitopes restricted by HLA-B*57 in TC 351 during the follow-up.

DISCUSSION

In this work, we showed that low Gag-specific T-cell polyfunctionality, high viral evolution, and high levels of proinflammatory cytokines were associated with the future loss of natural virological control. In addition, RANTES levels may be considered a potential novel biomarker predictive of the loss of virological control in EC.

The lack of HIV-1-specific T-cell responses is considered a hallmark of progressive HIV-1 infection (20). Preserved CD4$^+$ and CD8$^+$ T-cell multifunctional responses have been consistently associated with the control of viral replication in EC (4, 21, 22). However, this generalized assumption was derived from cross-sectional studies with heterogeneity between study subjects (23, 24). Our study demonstrates that TC show



Variable	Area	Error	P-value	95% CI Lower	95% CI Upper	Sensitivity (%)	Specificity (%)
Model A	0.972	0.035	0.001	0.825	0.977	100	75
Model B	1.000	0.000	0.001	1.000	1.000	100	100
Model C	1.000	0.000	0.001	1.000	1.000	100	100

FIG 8 Soluble cytokines and chemokines as potential biomarkers of the loss of virological control. Assays were performed with all time point samples in PC and with samples at preloss time points of follow-up (–T2 and –T1) in TC. (A) A fold change heat map of the relative plasma concentrations of measured inflammatory markers is shown. Positive folding (green) means higher concentrations in PC, while negative folding (red) means the opposite. Of these potential biomarkers, the five marked with an asterisk reached a concentration with a *P* value of <0.05 in the Mann-Whitney U test, and the remaining two were added to the list due to their classification power in the two-multivariate test. (B) Random forest analysis importance plot of the top 20 variables in importance of classification from a total of 70 cytokines and chemokines. Only the top five, highlighted in bold, were considered potential biomarkers. (C) The score plot of the PCA showed that the best percentage of separation between groups was achieved with only two variables, RANTES and PDGF AA. (D) Using logistic regression and receiver operator characteristic (ROC) curves, we assessed three different multimarker models that could accurately predict the loss of control in EC: model A (blue), which includes the statistically significant variables in the Mann-Whitney U test, model B (green), composed of the top five variables obtained from the random forest analysis, and model C (orange), compounded by the two variables obtained in the PCA analysis.

a low magnitude of Gag-specific T-cell responses together with fewer polyfunctional T cells than PC. In addition, this specific T-cell response has a particular mature and more highly activated phenotype in TC, in agreement with previous findings of T-cell activation preceding the viral breakthrough after spontaneous control in acute infection (25). These characteristics precede by 1 year the loss of spontaneous control. These results may explain the variability observed in previously studied cohorts (24, 26, 27) in which virological progression was not taken into account, and because of that, these studies could not discriminate between TC and PC. In addition, our data are in

agreement with a previous work showing a decreased $CD8^+$ T-cell breadth that was associated with loss of viral control but in viremic controllers, although we did not observe an association between functional Gag-specific $CD8^+$ T cells and the presence of protective HLA class I alleles (28). One possible mechanism involved in the loss of control may be related to the fact that $CD8^+$ T cells restricted by "nonprotective" HLA allele groups can be suppressed by regulatory T cells (29). Ferrando-Martinez et al. (26) showed that mature (EM) Gag-specific $CD8^+$ T cells from EC had higher polyfunctionality than viremic controllers and non-HIV-1 controllers. In our work, we observed increased polyfunctionality in more mature (TD $CD57^+$) Gag-specific $CD8^+$ T cells from PC but not from TC. This result suggests that Gag-specific $CD8^+$ T cells with a mature phenotype have increased cytotoxic abilities and points to this mechanism as one of the main causes of persistent natural control. The absence of differences in polyfunctional distribution between PC and TC in this subset at T0 may be explained by the particular mature and more highly activated phenotype of Gag-specific T-cell response and higher bulk T-cell activation at this time point. Regarding Gag-specific $CD4^+$ T cells, the dramatic decrease in this response 1 year before the loss of control is in agreement with a previous study showing the important role of simian immunodeficiency virus (SIV)-specific $CD4^+$ T cells in the breakthrough SIVmac239 viremia in an elite controller (30).

Among other viral properties, genetic variability and viral evolution have been associated with viral pathogenesis and disease progression (31–35). Proviral DNA from peripheral blood mononuclear cells (PBMCs) is considered to harbor a combination of recently produced and archived variants, and consequently, variability in this compartment better reflects viral evolution since primoinfection (18, 36). Analysis of *env* and *gag* proviral DNA sequences, in samples prior to the loss of control, showed evidence of viral divergence and diversity in TC, demonstrating not fully restricted viral replication despite undetectable VL. On the contrary, PC presented minimal or no proviral divergence and extremely low diversity, suggesting no viral replication since primoinfection or that replication is occurring at such a low rate that reseeding of this proviral compartment is not possible, as suggested in EC, although evolution in plasma virus was detected (37–39). Lower levels of viral diversity were observed in EC than in patients with undetectable levels of viremia due to combined antiretroviral treatment (39, 40). Moreover, the viral diversity in EC is not directed by neutralizing antibodies (40), although escape mutants to neutralizing antibodies are continuously generated and selected in these patients (41). Our results are in agreement with previous works showing a relation among viral replication and evolution, viral pathogenesis, and disease progression (33–35, 37, 42, 43). Therefore, markers of HIV-1 viral evolution (divergence and diversity) could differentiate the TC and PC phenotypes in the pool of EC. The homogeneous viral populations found in PC are also observed in viral infections with fidelity mutants of poliovirus (44, 45) or arbovirus (46), which result in less pathogenic infections (33, 34, 35).

Immunological and virological factors were intimately associated in these subjects. We found strong inverse associations between *gag* diversity and Gag-specific $CD8^+$ T-cell polyfunctionality (three functions) but only at the follow-up time points with undetectable viremia in TC. The association was found in *gag* and not in *env*, probably because we analyzed Gag- but not Env-specific T-cell responses. These observations are in agreement with previous findings that demonstrate that responses targeting *gag* but not *env* are inversely associated with VL (47, 48). The results presented herein revealed that the lack of viral evolution reflects the continuous immune pressure exerted by Gag-specific T-cell responses in PC and the fine balance between viral persistence and immune control in TC. Our results are in agreement with those of Noel et al., which demonstrated the contribution of abortive viral expression in $CD4^+$ T cells from EC for maintenance of strong HIV-1-specific $CD8^+$ T-cell responses in the absence of HIV-1 evolution (49). Likewise, these results may be in agreement with the work of Boritz et al. showing that clonal expansion of infected cells acts to maintain the virus in HIV controllers (18). The lack of viral evolution found in this work suggests that clonal

expansion may be one of the main mechanisms of virus persistence in PC. This mechanism may be enough for PC to maintain an enhanced Gag-specific T-cell response that is able to contain viral replication. On the other hand, a second mechanism proposed by Boritz et al. (18), consisting of replication of inducible proviruses of recent origin, may be the prevalent mechanism in TC before the loss of control, reflecting a less efficient Gag-specific T-cell response that is more compatible to what happened in viremic controllers.

The different patterns observed in viral diversity and DNA proviral load could be linked with different magnitudes and qualities of Gag-specific T-cell responses and with different levels of inflammatory mediators. There is increasing evidence that EC have increased levels of T-cell and myeloid activation and unique inflammatory signatures in comparison with HIV-suppressed and HIV-1-uninfected subjects (50–52). Interestingly, a lack of an association between inflammatory markers and VL in EC has been described before (13), suggesting that persistent viral replication may not be the main trigger behind the inflammation observed in EC. Our data support that despite no detectable plasma VL, the lower Gag-specific T-cell response in TC and higher viral diversity and DNA proviral load may be related to the higher levels of several inflammatory cytokines in TC.

This is the first extensive analysis of the expression profile of 70 plasma cytokines and chemokines in EC with different virological evolutions. Random forest analysis, PCA, and ROC curves which included the combination of the selected proinflammatory markers revealed that RANTES and PDGF-AA may be sufficient to clinically categorize PC and TC. Of special interest is the chemokine RANTES, the most remarkable biomarker for group discrimination, whose concentration was four times higher in TC than in PC. Despite the fact that RANTES, the natural CCR5 ligand, has been previously shown to prevent HIV infection (53), RANTES can also be considered a proinflammatory cytokine, and it has been associated with HIV disease progression (54–56). Higher levels of RANTES may reflect low-level residual viral replication in TC that could be associated with $CD8^+$ T-cell dysregulation by exhaustion (57). Our findings not only define RANTES as a reliable biomarker for a rapid screening of potential EC with virological loss of control but also suggest that the immunomodulation of RANTES is a therapeutic target in EC.

This study has disclosed the presence of two distinct groups of EC, and we have been able to find differences between these two EC phenotypes based on immunological and virological markers. These results demonstrate that HIV-1 controllers are a heterogeneous group of subjects with different characteristics and nomenclatures (58), as we (59) and others (60) previously suggested. Accordingly, it is crucial to establish a precise definition of the EC phenotype in order to identify the correlates of persistent spontaneous control in the search for the right model of functional remission. These new insights might help in the reconsideration of the current treatment guidelines that recommend antiretroviral treatment for all HIV-1 controllers (https://aidsinfo.nih.gov/guidelines).

The main limitation of this study was sample availability, which constrained the evaluation of additional immunological and virological factors. However, these subjects are extremely rare, and even so, we were able to have a follow-up with samples before and after the loss of control.

In summary, this study has allowed the identification of several immunological, virological, and proinflammatory cytokines that will help in the accurate definition of EC and in their clinical management. In addition, the identification of important factors for the persistent natural control of HIV replication could give new clues to achieve a long-term remission status or functional cure in HIV-1-infected patients.

MATERIALS AND METHODS

Study participants. EC were defined as subjects with three consecutive VL determinations under the detection limit (<50 HIV-1 RNA copies/ml) in the absence of antiretroviral treatment for at least 1 year of follow-up (7). Subjects were included based on frozen peripheral blood mononuclear cells (PBMCs) and plasma samples available in the Spanish HIV HGM BioBank belonging to the AIDS Research Network (61) and with data in the RIS Controllers Study Group Cohort (ECRIS) (8) (see File S1 in the supplemental material) based on the study design. Thirty-one EC were analyzed; 14 of these underwent loss of

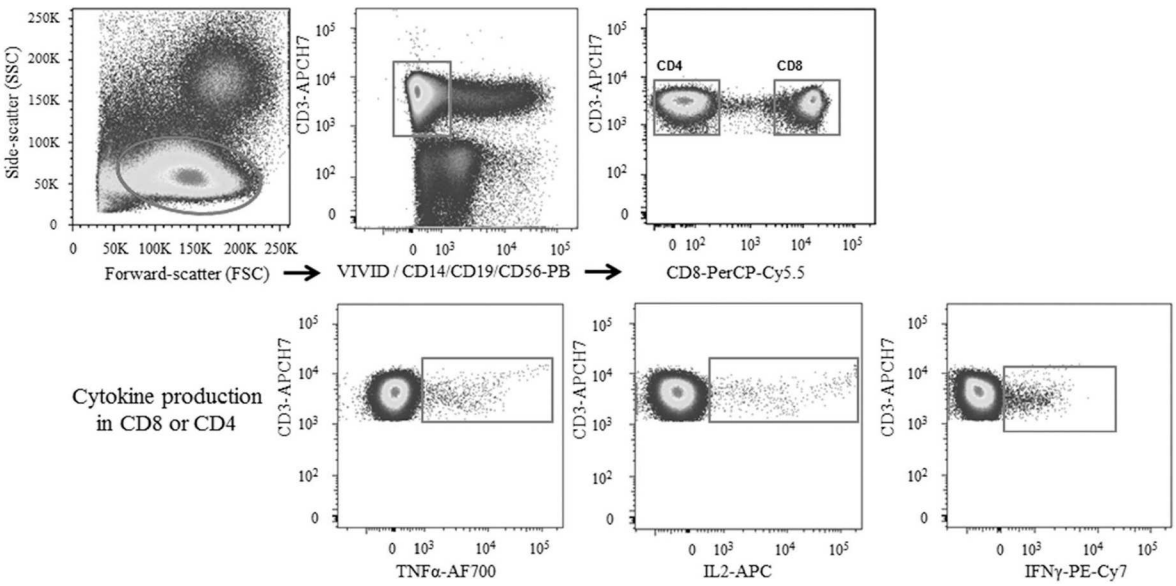


FIG 9 Schematic diagram of the cytometry gating strategy. For gating strategies for Gag-specific $CD4^+$ and $CD8^+$ T cells, the representative plots show the functional cytokine responses to Gag peptides.

virological control (at least two consecutive measurements of VL above the detection limit in 1 year) and were named transient controllers (TC). Seventeen EC who maintained persistent virological control during the same follow-up period were designated persistent controllers (PC) (see study design in Fig. 1). All subjects participating in the study gave their informed consent, and protocols were approved by the institutional ethical committees.

Experimental procedures. Laboratory evaluations were performed at the Laboratory of Immunovirology, Institute of Biomedicine of Seville (IBiS), Virgen del Rocío University Hospital in Seville (Spain); the Molecular Virology Unit, Laboratory of Research and Reference in Retrovirus, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid (Spain); the Joan XXIII University Hospital in Tarragona, IISPV, Rovira i Virgili University (Spain); and the AIDS Research Institute IrsiCaixa, Badalona (Spain).

General. Absolute counts of $CD4^+$ and $CD8^+$ T cells were determined in fresh whole blood by using an Epic XL-MCL flow cytometer (Beckman-Coulter, Brea, CA) according to the manufacturer's instructions. The plasma HIV-1 RNA concentration was measured by using quantitative PCR (Cobas Ampliprep/Cobas TaqMan HIV-1 test; Roche Molecular Systems, Basel, Switzerland) according to the manufacturer's protocol. The detection limit for this assay was 50 HIV-1 RNA copies/ml. Hepatitis C virus (HCV) RNA was determined using an available PCR procedure kit (Cobas Amplicor; Roche Diagnostics, Barcelona, Spain) with a detection limit of 10 IU/ml.

Genetics. HLA-B group alleles were genotyped using a reverse sequence-specific oligonucleotide bound to a fluorescently coded microsphere system (LABType SSO, RSSO1B; One Lambda, Canoga Park, CA) by following the manufacturer's instructions. The genotyping of the IL28B single nucleotide polymorphism (SNP) rs12979860 was performed as previously described (62), using a TaqMan 5′ allelic discrimination assay (Applied Biosystems, Foster City, CA).

Cell stimulation. PBMCs were thawed, washed, and stimulated *in vitro* with 2 μg/ml of an overlapped HIV (Gag)-specific peptide pool (NIH AIDS Reagent Program [https://www.aidsreagent.org/index.cfm]) and stained with conjugated monoclonal anti-CD107a-BV786 (clone H4A3; BD Biosciences, Franklin Lakes, NJ) at the beginning of incubation as previously described (26).

Immunophenotyping and intracellular cytokine staining. Stimulated PBMCs were washed and stained with LIVE/DEAD fixable violet dead cell stain (Life Technologies, CA, USA). The cells were then surface stained with anti-CD14-PB, anti-CD19-PB, anti-CD38-Qdot655 (Life Technologies), anti-HLA-DR-BV570 (clone L243), anti-CD56-PB (Biolegend, San Diego, CA), anti-$CD8^+$-PerCP-Cy5.5 (clone RPA-T8), anti-CD45RA-fluorescein isothiocyanate (FITC) (clone L48), anti-CD27-BV605, and anti-CD57-phycoerythrin (PE)-CF595 (BD Biosciences). Cells were then stained intracellularly for 30 min with 100 μl of phosphate-buffered saline (PBS) with anti-CD3-allophycocyanin (APC)-H7 (clone SK7), anti-IFN-γ-PCy7, anti-tumor necrosis factor alpha (TNF-α)-Alexa 700 (clone MAb11), anti-IL-2-APC (clone MQ1-17H12), and anti-perforin-PE (clone B-D48) (BD Biosciences) and then washed twice and fixed in PBS containing 4% paraformaldehyde (PFA). Unstimulated cells and cells stimulated with staphylococcal enterotoxin B (SEB) as a positive control were included in each experiment. Lymphocytes were defined as having low forward/side scatter and expressing CD3, and/or no CD8, but not CD19, CD14, and CD56 (Fig. 9).

PBMCs were analyzed by using a LSR Fortessa cell analyzer (BD Biosciences, Spain). A minimum of 1,500,000 total events were recorded for each condition.

Cytokine and chemokine measurement by Milliplex bead array kits. The compositions of Milliplex MAP human cytokine/chemokine magnetic bead panel kits were as follows. HCYTMAG-60K-PX23 included epidermal growth factor (EGF), IL-1α, fibroblast growth factor 2 (FGF-2), IL-3, eotaxin-1, IL-7, transforming growth factor alpha (TGF-α), IL-8, granulocyte colony-stimulating factor (G-CSF), IP-10, fractalkine, monocyte chemoattractant protein 1 (MCP-1), IFN-α2, MIP-1α, MIP-1β, GRO, MCP-3, vascular endothelial growth factor (VEGF), IL-12 (p40), Flt-3 ligand, MDC (CCL22), sCD40L, and IL-1ra.

HCYTMAG-60K-03 included PDGF-AA, PDGF-AB/BB, and RANTES.

HT17MG-14K-PX25 included granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-21, IFN-γ, IL-4, IL-17F, IL-23, IL-10, IL-5, CCL20/MIP-3α, IL-6, IL-13, IL17E/IL-25, IL-15, IL-27, IL17A, IL-31, IL-22, TNF-α, IL-9, TNF-β, IL-1β, IL28A, IL-33, IL-2, and IL-12 (p70).

HCP2MAG-62K-19 included eotaxin-2, IL-20, MCP-2, TRAIL, BCA-1, CTACK, MCP-4, SDF-1A+b, I-309, ENA-78, IL-16, MIP-1d, TARC, 6CKINE, eotaxin-3, LIF, TPO, SCF, and TSLP6.

Median fluorescence intensities were collected on a Bio-Plex 200 instrument by using Bio-Plex Manager (Bio-Rad Laboratories, Spain) software. Cytokine concentrations were determined from the appropriate standard curves to convert fluorescence units to concentrations (pg/ml).

Nucleic acid extraction and *gag* and *env* amplification. Proviral DNA was obtained from 2×10^6 frozen PBMCs using a Speedtools tissue DNA extraction kit (Biotools B&M Labs S.A., Spain). We used a limiting-dilution nested PCR using Phusion high-fidelity PCR master mix with HF buffer (Thermo Scientific). A first multiplex PCR for *gag* and *env* region amplification was performed using the outer primers 505-gag (5′ CGAGGGGCGGCGACTGGT 3′; HXB2 positions 728 to 745), 40-gag (5′ TTCCCTAAAA AATTAGCCTGTCT 3′; HXB2 positions 2074 to 2096), 169-env (5′ AATGTCAGCACAGTACAATGTACAC 3′; HXB2 positions 6945 to 6969), and 96-env (5′ AGACAATAATTGTCTGGCCTGTACCGT 3′; HXB2 positions 7836 to 7862). One microliter of the first PCR product was reamplified independently using primers for *gag*, i.e., 171-gag (5′ TTTGACTAGCGGAGGCTAG 3′; HXB2 positions 761 to 779) and 336-gag (5′ TTCCA ACAGCCCTTTTTCCTAGGGG 3′; HXB2 positions 2009 to 2033), or primers for the C2-V5 region, i.e., 27-env (5′ ATAAGCTTGCAGTCTAGCAGAAGAAGA 3′; HXB2 positions 7004 to 7030) and 167-env (5′ TTCTCCAA TTGTCCCTCATATCTCCTCCTCCA 3′; HXB2 positions 7634 to 7665). Nucleotide sequences were determined with the Big DyeTM terminator cycle sequencing kit (Applied Biosystems) in an ABI 3730 sequencer (Applied Biosystems) in the Genomic Unit of the CNM-ISCIII.

Proviral DNA quantification. The DNA proviral viral load was quantified by using a nested *Alu*-long terminal repeat (Alu-LTR) PCR (63, 64). In brief, a first conventional PCR was performed using oligonucleotides against *Alu* sequence and the HIV-1 LTR, with the following conditions: 95°C for 8 min and then 12 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 10 min, followed by 1 cycle of 72°C for 15 min. Then, a second quantitative PCR was performed using TaqMan probes with FAM/ZEN/Iowa Black and TaqMan master mix (Applied Biosystems). DNA from the 8E5 cell line was used for the standard curve. The *ccr5* gene was used as a housekeeping gene for measuring the input DNA and to normalize the data.

Phylogenetic analysis of the nucleotide sequences in *gag* and *env* genes. Nucleotides sequences were assembled using the SeqMan program (DNASTAR). Maximum likelihood trees were constructed as follows. First, nucleotide sequences were included in a global database with the sequences obtained from the patients studied in our laboratory to detect cross-contamination. All samples segregated in distinct clusters of the phylogenetic tree, excluding contamination. Phylogenies were estimated by a maximum likelihood approach using the best-fit model of nucleotide substitution (GTR+G+I; jModelTest v.0.1.1) implemented in the MEGA 6 software program (65). Internal branch support was tested with an approximate likelihood ratio test (MEGA 6). Intrasample diversity was estimated by the best-fit model of nucleotide substitution in the MEGA 6 program. An estimation of viral infection time or "viral dating" was deduced, assuming a relaxed molecular clock, from the genetic distance of the nucleotide sequence of each subject virus to the MRCA (most recent common ancestor) of the HIV-1 Spanish epidemic (6, 10, 11, 66).

Analysis of Gag CD8$^+$ T-cell immune escape. HIV-1 variation at the optimal Gag CD8$^+$ T-cell epitopes was based on the best-defined CD8$^+$ epitope summary from the Los Alamos Molecular Immunology Database in Gag proviral sequences matched to patients' HLA class I alleles with two- to four-digit resolution (35). HIV-1 variation at optimal epitopes was defined by comparison with the HIV-1 HXB2 epitope sequence at the HLA-I restriction element. The frequency of virus variation at Gag epitopes was defined as the ratio of total mismatches found at optimal epitopes to the number of total optimal epitopes found per sequence.

Statistical analysis. Correlations between variables were assessed using the Spearman rank test. Differences between categorical values were determined by the chi-square test. Differences between unpaired groups were determined by the Mann-Whitney U test, and differences between paired samples were determined by the Wilcoxon signed rank and Friedman tests. P values of <0.05 were considered statistically significant. The Statistical Package for the Social Sciences software (SPSS) 22.0 package (IBM, Madrid, Spain) was used for the statistical analysis. Graphs were generated with Prism, version 5.0 (GraphPad Software, Inc.). Polyfunctionality was defined as the percentage of lymphocytes producing multiple cytokines. Polyfunctionality pie charts were constructed using Pestle version 1.6.2 and Spice version 5.2 (provided by M. Roederer, NIH, Bethesda, MD) and was quantified with the polyfunctionality index algorithm (67) employing the 0.1.2 beta version of the FunkyCells Boolean Dataminer software provided by Martin Larson (INSERM U1135, Paris, France).

The selection of potential biomarkers among the soluble markers of inflammation associated with the loss of spontaneous control was complemented with multivariate statistics using random forest analysis (unbiased selection of variables) and principal-component analysis (PCA) (multivariate pattern that generates the maximum degree of separation between groups), and finally, the performance of biomarkers was examined by logistic regression analysis and receiver operating characteristic (ROC)

curves. The employed statistical software included the R software (http://cran.r-project.org), matrix calculation platform MATLAB (version 7.5.0; The Mathworks, Inc., Natick, MA, USA).

Accession number(s). Nucleotide sequences were submitted to GenBank under accession numbers MF988754 to MF989105.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JVI.01805-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We declare that no conflicts of interest exist.

E.R.-M. and M.L. designed the study. N.R., J.M.B., A.L., M. Planas, F.G., C.R., O.M.-M., M.D., J.A.I., J.D.R., M.M.-F., F.V., J.A., and M.L. coordinated the patient inclusion and sample management and analyzed and interpreted data from the experiments. M. Pernas, C.C., L.T.-D., C.L.-G., E.R.-M., J.G.-P., and M.C. designed the experiments. M. Pernas, I.O., L.T.-D., B.D.-M., E.R.-G., J.G., J.G.-P., and M.C. produced the experimental data in the laboratory. M. Pernas, L.T.-D., E.R.-G., C.L.-G., and E.R.-M. prepared the manuscript. All authors contributed to reviews of the manuscript.

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MAJOR ARTICLE

Proteomic Profile Associated With Loss of Spontaneous Human Immunodeficiency Virus Type 1 Elite Control

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Background. Elite controllers (ECs) spontaneously control plasma human immunodeficiency virus type 1 (HIV-1) RNA without antiretroviral therapy. However, 25% lose virological control over time. The aim of this work was to study the proteomic profile that preceded this loss of virological control to identify potential biomarkers.

Methods. Plasma samples from ECs who spontaneously lost virological control (transient controllers [TCs]), at 2 years and 1 year before the loss of control, were compared with a control group of ECs who persistently maintained virological control during the same follow-up period (persistent controllers [PCs]). Comparative plasma shotgun proteomics was performed with tandem mass tag (TMT) isobaric tag labeling and nanoflow liquid chromatography coupled to Orbitrap mass spectrometry.

Results. Eighteen proteins exhibited differences comparing PC and preloss TC timepoints. These proteins were involved in proinflammatory mechanisms, and some of them play a role in HIV-1 replication and pathogenesis and interact with structural viral proteins. Coagulation factor XI, α-1-antichymotrypsin, ficolin-2, 14-3-3 protein, and galectin-3-binding protein were considered potential biomarkers.

Conclusions. The proteomic signature associated with the spontaneous loss of virological control was characterized by higher levels of inflammation, transendothelial migration, and coagulation. Galectin-3 binding protein could be considered as potential biomarker for the prediction of virological progression and as therapeutic target in ECs.

Keyword. biomarkers; elite controllers; HIV-1; loss of control; proteomic profile.

The so-called elite controllers (ECs), who represent a minority group of subjects in the scenario of human immunodeficiency virus type 1 (HIV-1) infection (~1%), are able to maintain an undetectable viral load (VL) in the absence of combined antiretroviral therapy (ART) [1]. Due to this characteristic, ECs have been proposed as a model of functional cure, eradication strategies, and also HIV vaccine development [2]. Knowledge of the mechanisms involved in the controller phenomenon is highly relevant for the identification of the virological and host determinants involved in the spontaneous control.

Host genetic factors, mainly human leukocyte antigen (HLA) class I molecules, such as HLA-B*57 [3], as well as immunological mechanisms, such as the HIV-1–specific T-cell response characterized by increased production of cytokines, chemokines, and cytolytic enzymes (named polyfunctionality), and HIV-1 suppression capacity [4–6] have been associated with this clinical situation.

New evidence suggests that these subjects have heterogeneous clinical outcomes including a variable proportion who lose HIV control overtime [7, 8]. Although recent findings have identified transient controllers (TCs) as subjects with low Gag-specific T-cell polyfunctionality, high viral diversity [9], high proinflammatory cytokine levels [7], and T-cell homeostasis disturbances [10], to date, no longitudinal study elucidating the proteomic profile associated with the loss of spontaneous HIV-1 elite control has been performed. In the HIV infection scenario, proteomics has emerged for identifying proteins involved in virus pathogenesis in several models of HIV-1 disease [11]. A better understanding of HIV-1 and human protein interactions might be used as a starting point for further functional analysis that may also be translated into novel therapeutic strategies [12].

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Thus, the aim of this work was to study the plasma proteomic profile that preceded the loss of spontaneous virological control in ECs to identify the involved mechanistic pathways that can be further explored to find the potential interventional targets and also to identify the potential biomarker predictors of virological progression in these subjects.

METHODS

Patients and Study Design

EC were defined as subjects with VL determinations below the detection limit (<50 HIV-1 RNA copies/mL) in the absence of ART for at least 12 months [13]. Subjects were included based on frozen plasma sample availability according to the study design (Supplementary Figure 1). Samples were received, processed, and stored at the Spanish HIV Hospital General Universitario Gregorio Marañón BioBank belonging to the AIDS Research Network (RIS) [14], and data were registered in the RIS cohort of the HIV Controllers Study Group (ECRIS) (Supplementary Data). The cohort's characteristics were previously described in detail [8, 9, 13]. A total of 16 subjects were retrospectively selected and analyzed: 8 were ECs who had experienced loss of spontaneous virological HIV-1 control (at least 2 consecutive measurements of VL above the detection limit over 12 months), named TCs, and another group of 8 ECs who persistently maintained virological control during the same follow-up period, named persistent controllers (PCs) (see study design in Supplementary Figure 1). In TCs, up to 4 determinations were assessed: at 1 and 2 years before the loss of virological control (–T1 and –T2, respectively), called the "pre–loss-of-control period," and up to 2 more in the "post–loss-of-control period" including the closest time point (T0) and 1 year (T1) after the loss of virological control. In PCs, up to 2 determinations were performed at 1-year intervals.

We also assessed 3 other subsets of individuals: uninfected controls, viremic HIV-infected patients with progressive disease, and virologically suppressed HIV-infected patients on ART (see "Western blot" section). Samples were stored at the Institut d'Investigació Sanitària Pere Virgili BioBank following standard procedures and with the appropriate approval of the ethical and scientific committees.

All subjects provided their informed consent, and the protocols were approved by the institutional ethical committees. Detailed information about laboratory procedures can be found in the Supplementary Data.

Proteomic Analysis

For proteomic analysis, the 7 most abundant plasma proteins (albumin, immunoglobulin G [IgG], antitrypsin, immunoglobulin A [IgA], transferrin, haptoglobin, and fibrinogen) were depleted using a human-7 multiple affinity removal spin column cartridge (Agilent) following the manufacturer's protocol, and the flow-through fractions were concentrated and buffer exchanged to approximately 100 µL of 50 mM ammonium bicarbonate using 5K molecular weight cut-off spin columns (Agilent). This immunoaffinity depletion enhances the detection of lower abundance proteins and improves the subsequent analysis of serum samples expanding the dynamic range of the analysis.

Then, 65 µg of protein (quantified by Bradford method) were incubated at 96°C for 3 minutes, reduced with 4 mM 1.4-dithiothreitol for 25 minutes at 56°C, and alkylated with 8 mM iodoacetamide for 30 minutes at 25°C in the dark. Afterward, samples were digested overnight (pH 8.0, 37°C) with sequencing-grade trypsin (Promega) at an enzyme:protein ratio of 1:50. Peptides were desalted on a C18 SPE column (BondElut, Agilent) and labeled with tandem mass tag (TMT) 10-plex reagents (Thermo Fisher) following the manufacturer's instructions. To normalize all samples in the study, a pool containing all of the samples was labeled with TMT-126 tag and included in each TMT batch.

Multiplexed samples were on-line fractionated in a strong cation exchange nanocolumn (350 µm × 3.5 cm; 3.5 µm, Agilent) coupled to a C18 trap nanocolumn (100 µm × 2 cm; 5 µm, Thermo Fisher) and a C-18 analytical nanocolumn (75 µm × 15 cm; 3 µm, Nikkyo Technos) on an EASY-II nanoLC chromatograph (Thermo Fisher) by a gradient salt pulsed sequential elution using ammonium acetate (0, 12.5, 25, 37.5, 50, 75, 100, 250, and 500 mM ammonium acetate) followed by a continuous water-acetonitrile (0.1% formic acid) elution gradient at 300 nanoliters (nL)/minute.

Mass spectrometry analyses were performed on an LTQ-Orbitrap Velos Pro (Thermo Fisher) by acquiring an enhanced fourier transformation (FT)-resolution spectrum (R = 30 000 full width at half maximum [FHMW]) followed by 2 data-dependent tandem mass spectrometry (MS/MS) scan events from the most intense 10-parent ions with a charge state rejection of 1 and a dynamic exclusion of 0.5 minutes. Thus, an higher-energy collisional dissociation fragmentation (40% normalized collision energy [NCE]) with FT-MS/MS acquisition (R = 15 000 FHMW) was conducted for peptide quantification, followed by a collision induced dissociation fragmentation (35% NCE) from the same parent ions with ion trap (IT)-MS/MS acquisition for peptide identification.

Protein identification/quantification was performed on Proteome Discoverer software version 1.4.0.288 (Thermo Fisher) by multidimensional protein identification technology. For protein identification, the MS and MS/MS spectra were analyzed using Mascot search engine (version 2.5) with SwissProt_2016_07.fasta database (551 705 entries), restricted for human taxonomy (20 198 sequences) and assuming trypsin digestion. Two missed cleavages and an error of 0.02 Da for FT-MS/MS, 0.8 Da for IT-MS/MS, and 10.0 ppm for a FT-MS spectra were allowed. The TMT-10-plex was set as quantification modification, oxidation of methionine and acetylation of N-termini were set as dynamic modifications, whereas carbamidomethylation of cysteine was set as static modification. The false discovery rate and protein probabilities were calculated by Percolator.

For protein quantification, the ratios between each TMT label against the 126-TMT label were used, and the quantification results were normalized based on the protein median to reduce experimental bias and $\log_2$ transformed and mean centered for variance stabilization, data range compression, and making the data more normally distributed before statistical analysis. Detailed information about laboratory procedures can be found in the Supplementary Data. In addition, Supplementary Figure 2 shows concentration distribution graph and principal components analysis (PCA) before and after normalization steps.

Western Blot

Protein samples were submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membrane was then blocked, incubated with a primary antibody followed by an appropriate secondary antibody conjugated with horseradish peroxidase, and developed by chemiluminescence using Versadoc (Bio-Rad Laboratories). Finally, the proteins were quantified by ImageJ software.

The primary antibodies used were LGALS3BP (ab67353), ficolin-2 (ab56225), anti-α 1 antichymotrypsin (EPR14118[B]), and factor XI (MM0193-7C38) (all from Abcam) and 14-3-3 ζ/δ (catalog number 7413, Cell Signaling Technology). Secondary antibodies were polyclonal goat antirabbit (Pierce) and polyclonal goat antimouse (Pierce). Ponceau S staining was used as loading control.

We also compared the levels of the proteins of interest in PC and TC individuals to the levels seen in uninfected controls, viremic individuals with progressive disease, and virologically supressed HIV-infected patients on ART (n = 8 of each group) to see if the protein profiles in TCs were more similar to those of viremic individuals.

Protein Function and Pathway Analysis

Protein function was elucidated by a Swiss-Prot database (http://www.expasy.org) search. Interaction between the differentially expressed proteins and HIV proteins was investigated with the HIV interaction database (http://www.ncbi.nlm.nih.gov/RefSeq/HIVInteractions) [15]. Signaling pathway analysis was defined with STRING version 10 (http://string.embl.de/) using proteins identified in this work and data from the Swiss-Prot function annotation as input [16].

Statistical Analysis

To find the significant protein changes between the different conditions, Mass Profiler Professional software version 14.5 (Agilent Technologies) was used. For statistical purposes, only those proteins that were quantified in >70% of the samples and in at least one of the groups were considered and protein quantification was normalized based on protein median and $\log_2$ transformed. First, a paired Student *t* test for PC samples was performed to check if a time effect existed in this group. Due to the lack of change in proteins in the PC group, we simplified the following analysis to a single variable, which is expressed as the mean value of 2 consecutive longitudinal determinations. Next, a Student *t* test between PCs and TCs in the pre–loss of control period was performed to find early biomarkers of control loss. In this sense, we focused on pre–loss of control timepoints from TCs and compared them with PCs. For all of the comparisons, a mean value of –T1 and –T2 was calculated. Finally, a paired *t* test between pre– and post–loss of control period in the TC group was conducted to find protein changes over time. In all of the comparisons, a multivariate analysis, such as hierarchical clustering analysis and PCA, was performed. To select and evaluate the performance of the potential biomarker, random Forest analysis, PCA, and characteristic operating characteristic (ROC) curves were conducted using the R program (http://cran.r-project.org) and the SPSS 21.0 package (IBM). *P* values <.05 were considered statistically significant.

RESULTS

Characteristics of the Studied Subjects

Clinical and demographic characteristics of the ECs at baseline are shown in Table 1. No differences were observed in age, sex, transmission route, or hepatitis C virus (HCV) coinfection; in $CD4^+$ or $CD8^+$ T-cell counts; or in the CD4:CD8 ratio between the TCs and PCs. The VL evolution after that time of follow-up is only partially known because some of these patients were rapidly treated with antiretrovirals. The median VL from TCs was 539 (interquartile range [IQR], 295–1120) HIV RNA copies/mL at T0 and 2740 (IQR, 985–22 250) HIV RNA copies/mL at T1. No comorbidities such as cardiovascular diseases, malignancies, hepatic diseases, metabolic disorders, bacterial pneumonia, renal diseases, or osteonecrosis were observed in any patient during the follow-up. Moreover, injecting drug users abandoned this habit and they were not under methadone treatment. Finally, the TC group presented a shorter time since diagnosis than PCs (3 [IQR, 2–8] years vs 13 [IQR, 10–17] years; *P* = .005). No differences were found in HLA-B frequencies.

Proteomic Profile Preceding the Loss of Natural HIV-1 Control in Transient Controllers

Using a shotgun proteomics approach, we were able to identify and quantify a total of 293 proteins in plasma samples. To evaluate protein changes as an effect of time in the PC group, we applied PCA analysis and a Student *t* test in paired samples in that group. No proteins significantly differed along time in PCs (Supplementary Figure 3).

As initial information regarding all of the quantified proteins, a partial least squares-discriminant analysis (PLS-DA) applied before statistical analysis is shown in Supplementary Figure 4. The PLS-DA model demonstrated that there was a great difference between PCs and TCs, using the complete result quantified set of proteins that defined the model with an accuracy of 100%, which suggested that some of these quantified proteins may become a potential biomarker for the selected conditions.

Table 1. Patient Characteristics

Characteristic	Transient Controllers (n = 8)	Persistent Controllers (n = 8)	*P* Value
Age, y, median (IQR)	41 (34–60)	44 (40–46)	.635
Male sex, No. (%)	5 (62.5)	4 (50)	.614
Transmission route, IDU, No. (%)	3 (37.5)	4 (50)	.198
Time since diagnosis, y, median (IQR)	3 (2–8)	13 (10–17)	**.005**
HCV RNA detected, No. (%)	3 (37.5)	3 (37.5)	.999
$CD4^+$ T cells/μL, median (IQR)	676 (623–963)	724 (609–985)	.817
$CD8^+$ T cells/μL, median (IQR)	787 (553–1162)	636 (432–1026)	.482
CD4:CD8 ratio, median (IQR)	0.86 (0.53–1.55)	1.08 (0.93–1.47)	.406
HLA B57, No. (%)	3 (37.5)	1 (12.5)	.248
HLA B27, No. (%)	1 (12.5)	1 (12.5)	.999
HLA B35, No. (%)	0 (0)	0 (0)	.999

Values from transient controllers are taken from 2 years before loss of virological control. Values are given as No. (%) for categorical variables or median (IQR) for continuous variables. The Mann–Whitney *U* test and χ^2 test were used. All *P* values <.05 were considered significant and are highlighted in bold.

Abbreviation: HCV, hepatitis C virus; IDU, intravenous drug use; IQR, interquartile range.

Eighteen proteins were found to exhibit statistically significant differences in plasma levels between TCs before the loss of HIV-1 control and PCs, within the complete set of important proteins defining the model (Table 2). As illustrated in the heatmap representation (Figure 1A), 7 of them were downregulated and 11 were upregulated in TCs compared with PCs. These proteins also showed good clusterization (Figure 1B) and a good differentiation between the studied groups (Figure 1C). In addition, a reliable separation between TCs' follow-up time points with those of PCs could be observed (Figure 1C), albeit there was some clustering between 4 samples of the PC T2 with TC –T1. All together, these data indicate the feasibility of these proteins to be potential biomarkers for the loss of HIV-1 control.

STRING was used to analyze specific pathways and protein networks involving the differentially expressed proteins for biological interpretation. This tool determined 2 related inflammatory signaling pathways: leukocyte transendothelial migration and complement and coagulation cascade (Figure 1D). As shown in Figure 1D, the host proteins galectin-3-binding protein (LG3BP) and ficolin-2 (FCN2) were found to interact with *gp120* (HIV function protein) and were upregulated (fold change [FC], 2 and 1.59, respectively), whereas intercellular adhesion molecule 1 (ICAM1), which interacts with *tat* protein, was downregulated in TCs before the loss of natural HIV-1 control (FC, 1.34).

To select and evaluate the performance of the potential biomarkers, we conducted a random Forest analysis (Figure 2A),

Table 2. List of the Differential Plasma Protein Levels Comparing Persistent Controllers and Transient Controllers at Pre–Loss of Control Timepoint

Swiss-Prot Accession No.	Swiss-Prot Entry	Protein Description	Gene Name	*P* Value
Q08380	LG3BP_HUMAN	Galectin-3-binding protein	*LGALS3BP*	2.83 (10–4)
P03951	FA11_HUMAN	Coagulation factor XI	*F11*	2.59 (10–3)
Q15485	FCN2_HUMAN	Ficolin-2	*FCN2*	4.53 (10–3)
P01011	AACT_HUMAN	α-1-antichymotrypsin	*SERPINA3*	1.06 (10–2)
P63104	1433Z_HUMAN	14-3-3 protein zeta/delta	*YWHAZ*	1.21 (10–2)
Q96IY4	CBPB2_HUMAN	Carboxypeptidase B2	*CPB2*	1.21 (10–2)
Q86UX7	URP2_HUMAN	Fermitin family homolog 3	*FERMT3*	1.93 (10–2)
O43866	CD5L_HUMAN	CD5 antigen-like	*CD5L*	2.47 (10–2)
P07360	CO8G_HUMAN	Complement component C8 gamma chain	*C8G*	3.05 (10–2)
P02753	RET4_HUMAN	Retinol-binding protein 4	*RBP4*	3.32 (10–2)
P35527	K1C9_HUMAN	Keratin, type I cytoskeletal 9	*KRT9*	3.33 (10–2)
P18206	VINC_HUMAN	Vinculin	*VCL*	3.62 (10–2)
Q6P4Q7	CNNM4_HUMAN	Metal transporter CNNM4	*CNNM4*	3.69 (10–2)
P60709	ACTB_HUMAN	Actin, cytoplasmic	*ACTB*	3.92 (10–2)
P05362	ICAM1_HUMAN	Intercellular adhesion molecule 1	*ICAM1*	3.99 (10–2)
P04114	APOB_HUMAN	Apolipoprotein B-100	*APOB*	4.05 (10–2)
P17936	IBP3_HUMAN	Insulin-like growth factor-binding protein 3	*IGFBP3*	4.23 (10–2)
P02775	CXCL7_HUMAN	Platelet basic protein	*PPBP*	4.73 (10–2)

Differences between groups were calculated using Student *t* test.

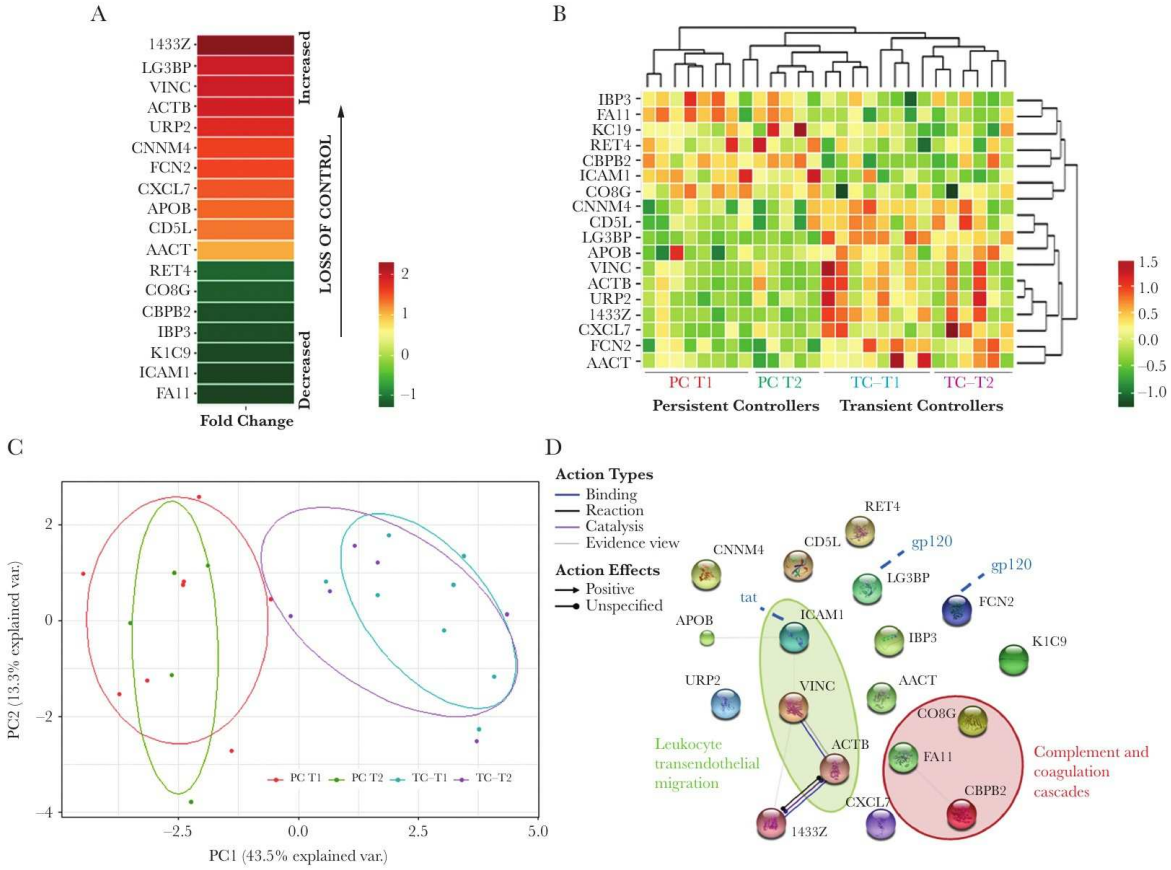


Figure 1. Proteomic analysis comparing transient controllers (TCs) before the loss of natural human immunodeficiency virus type 1 (HIV-1) control and persistent controllers (PCs), and a heatmap representation of the fold change of each quantified statistically significant proteins (*A*). Fold change was calculated as A/B − 1, where A was the variable mean in the PC group and B was the variable mean in the TC group. The scale from green (low abundance) to red (high abundance) represents the normalized abundance in arbitrary units. Hierarchical combined tree showing the clusterization of proteins (*B*) and PCA showing that these proteins allow differentiation between the studied groups and also between different time points (*C*): PCs first timepoint of the follow-up (T1; red, n = 8), PCs second timepoint of the follow-up (1-year interval) (T2; green, n = 5) (T2; green, n = 5), TCs 1 year before loss of virological control (–T1; blue, n = 8), TCs 2 years before loss of virological control (–T2; purple, n = 6). STRING network analysis of differentially expressed proteins in TCs before the loss of natural HIV-1 control and host–virus protein interactions (D): Line colors among proteins represent the interactions according to the legend (top left). The line symbol indicates the directionality of the effect in the case that it is known. The abbreviations for proteins are listed in Table 2. Abbreviations: PC, persistent controller; –T1, 1 year before loss of virological control; –T2, 2 years before loss of virological control; T1, first timepoint of the follow-up; T2 second timepoint of the follow-up (1-year interval); TC, transient controller.

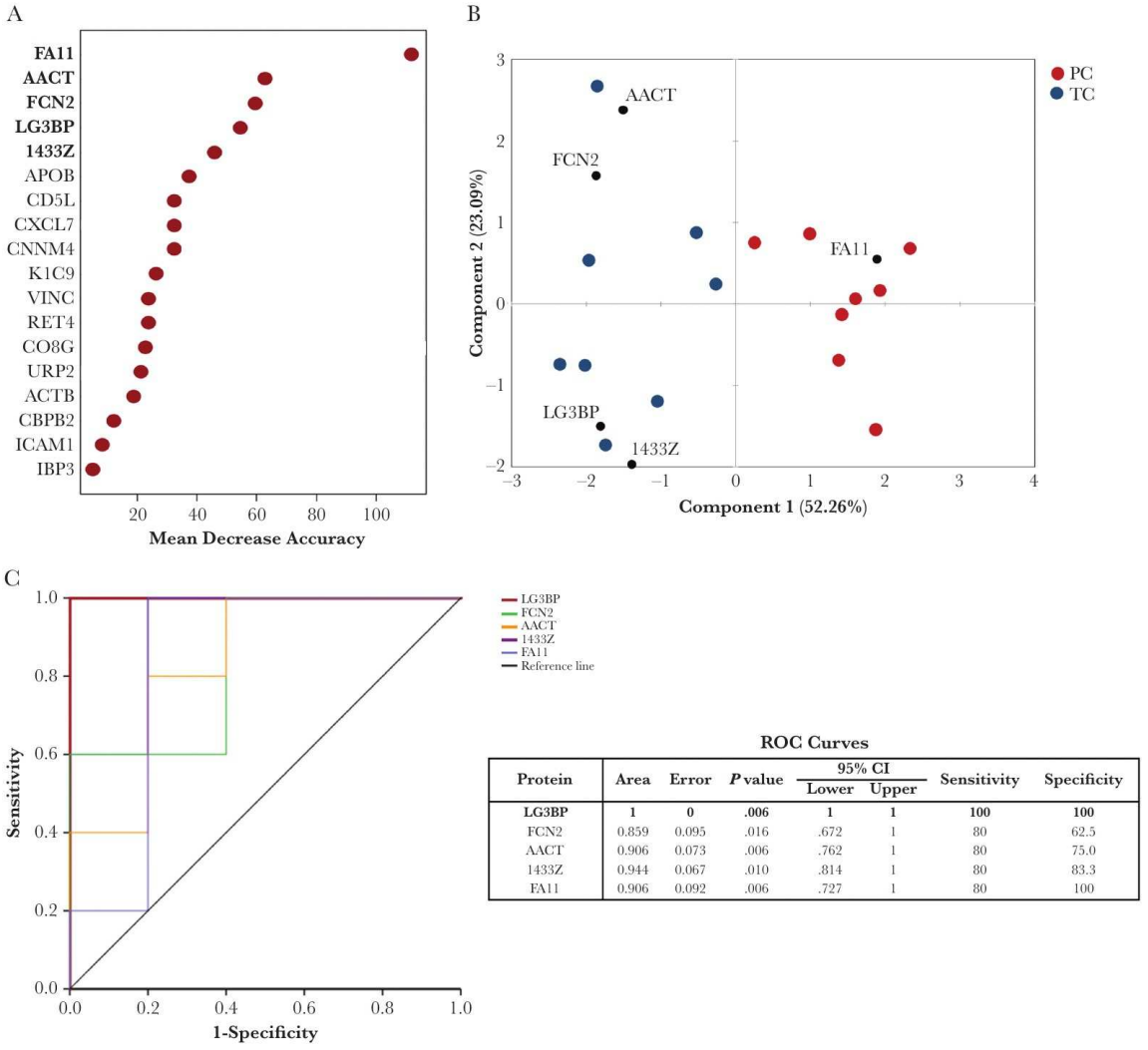
which elucidated that coagulation factor XI (FA11), α-1-antichymotrypsin, FCN2, LG3BP, and 14-3-3 protein zeta/delta (1433Z) were the main differentiators in a ranked list of the most significant proteins in order of their classification capability. It is important to highlight that these proteins were also the most significant variables in the univariate test.

After PCA analysis with these 5 proteins, a good differentiation between both groups was observed, which corroborates their predictive strength of virological progression in ECs (Figure 2B). Using logistic regression and ROC curves, although all of them have a statistically significant area under the curve, only LG3BP could discriminate PC and TC patients with 100% sensitivity and specificity, which suggests that this protein could be the most reliable biomarker for the prediction of the spontaneous loss of control in ECs (Figure 2C).

Finally, as shown in Supplementary Figure 5, there were not any differences in these proteins before and after the loss of control in TC group, so we could dismiss that the virus is changing the protein profile after the change of status.

Validation by Western Blot

Western blot analyses were performed to verify differentially expressed proteins between PC and TC in the pre–loss of control period and in searching for potential biomarkers. For this ascertainment, we selected the 5 main protein differentiators mentioned before (Figure 3A). The immunoblotting



ROC Curves

Protein	Area	Error	*P* value	95% CI		Sensitivity	Specificity
				Lower	Upper		
LG3BP	**1**	**0**	**.006**	**1**	**1**	**100**	**100**
FCN2	0.859	0.095	.016	.672	1	80	62.5
AACT	0.906	0.073	.006	.762	1	80	75.0
1433Z	0.944	0.067	.010	.814	1	80	83.3
FA11	0.906	0.092	.006	.727	1	80	100

Figure 2. Proteins as potential biomarkers of the loss of virological control. Random Forest analysis plot of the 18 proteins ordered by importance of classification. Only the top 5 were considered potential biomarkers (*A*). Score plot of the principal components analysis using this top 5 entities showed good differentiation between transient controllers (blue, n = 8) and persistent controllers (red, n = 8) (*B*). Logistic regression and receiver operating characteristic curves elucidated galectin-3 binding protein as the most reliable potential biomarker for the prediction of the spontaneous loss of virological control in elite controllers (*C*). The abbreviations for proteins are listed in Table 2. Abbreviations: CI, confidence interval; PC, persistent controller; ROC, receiver operating characteristic; TC, transient controller.

results confirmed the observations of the shotgun proteomic approach: LG3BP, FA11, and 1433Z showed the highest differences between groups. LG3BP and 1433Z were upregulated in TCs, whereas FA11 was downregulated in this group of ECs (Figure 3B). We also confirmed that, as suggested by the previous analyses, LG3BP showed the greatest difference between groups.

Finally, when we compared the levels of these 5 proteins to the levels in uninfected controls, viremic individuals with progressive disease, and individuals on ART, we could see that TC and viremic HIV-infected patients showed a similar trend in most cases. However, protein profiles in PC were comparable to uninfected patients and by some manner to virologically supressed HIV-infected patients on ART (Figure 4).

Protein Changes in Transient Controllers as an Effect of the Loss of Control

We also determined protein changes in TCs as an effect of detectable viremia; in other words, we wanted to see if there was any change between pre– and post–loss of control. In this comparison, 9 proteins showed statistically significant differences in plasma levels between TC pre-loss and post-loss timepoints

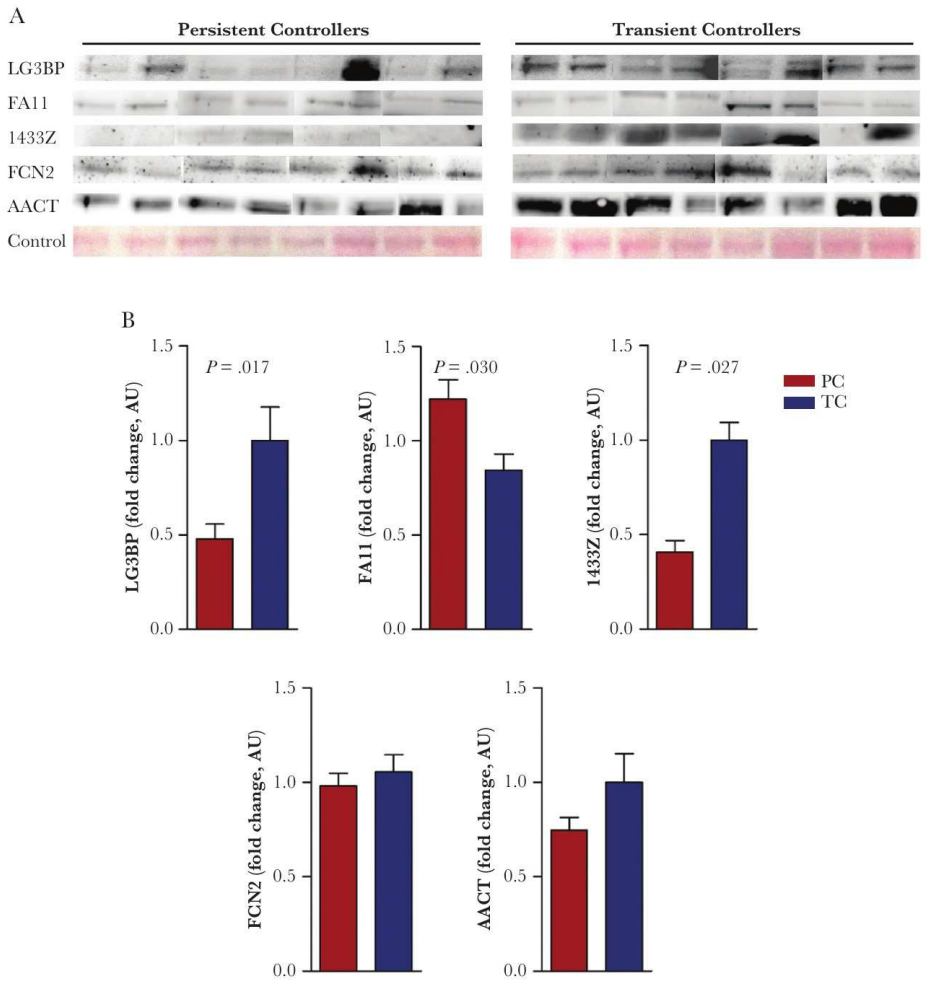


Figure 3. Protein validation by Western blot analyses. Representative immunoblot of selected proteins in persistent controllers or transient controllers using Ponceau S staining as loading control (*A*). Changes in protein levels between individuals were calculated by immunoblot densitometry; n = 8 patients from each group (*B*). Abbreviations: 1433Z, 14-3-3 protein zeta/delta; AACT, α-1-antichymotrypsin; AU, arbitrary units; FA11, coagulation factor XI; FCN2, ficolin-2; LG3BP, galectin-3-binding protein; PC, persistent controller; TC, transient controller.

(Supplementary Table 1). The heatmap representation showed that 5 proteins were upregulated after the loss of control, whereas 4 proteins were downregulated after the loss of virological HIV-1 control (Figure 5A). Of note, SH3 domain-binding glutamic acid rich–like protein expression was almost 3-fold higher after the loss of control (Figure 5B).

Interestingly, the pathway analysis showed that 3 of these proteins were involved in complement activation, which suggests an impaired regulation of that signaling pathway (scheme in Supplementary Figure 6).

DISCUSSION

In this study, we analyzed the proteomic profile of plasma associated with the virological progression of EC. A specific proteomic signature that included mainly proteins involved in proinflammatory pathways identified subjects who were going to lose natural HIV-1 control. Thus, these proteins could be considered potential biomarkers to rapidly screen for future loss of natural control as well as members of mechanistic pathways to be further explored to discover potential drug targets in ECs for achieving persistent control.

This is the first extensive analysis of the proteomic profile in plasma in ECs with different viral load evolution. From the 18 proteins differentially expressed before the loss of natural control in TCs compared to PCs, 3 were related with the cytoskeleton: keratin type I cytoskeletal 9, vinculin (VINC), and actin (ACTB). ACTB is involved in HIV-1 pathogenesis, including entry [17], viral assembly and budding [18, 19], and cell-to-cell

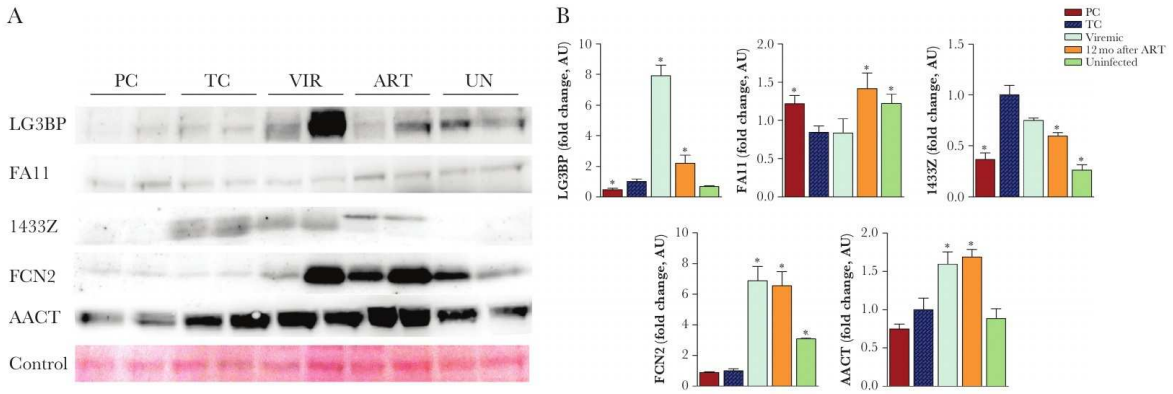


Figure 4. Representative immunoblot of selected proteins using Ponceau S staining as loading control (*A*). Changes in protein levels between individuals were calculated by immunoblot densitometry; n = 8 patients from each group (*B*). **P* < .05 compared with transient controllers. Abbreviations: 1433Z, 14-3-3 protein zeta/delta; AACT, α-1-antichymotrypsin; ART, virologically supressed human immunodeficiency virus–infected patients on antiretroviral therapy; AU, arbitrary units; FA11, coagulation factor XI; FCN2, ficolin-2; LG3BP, galectin-3 binding protein; PC, persistent controller; TC, transient controller; UN, uninfected individuals; VIR, viremic human immunodeficiency virus–infected patients with progressive disease.

HIV-1 transmission [20]. In myeloid cells [21], HIV-1 *Nef* protein induced a marked polarization of VINC, which directly binds ACTB and is overexpressed in apoptotic cells [22]. VINC knockdown has also been related to increased rates of infection [23] and has been considered a potential biomarker for HIV-1-infection [24]. The higher abundance of VINC and ACTB found in TCs might reflect low residual viral replication levels and cell-to-cell HIV-1 transmission. In addition, VINC and ACTB, as well as ICAM1, have also been shown to be involved in leukocyte transendothelial migration, which suggests an inflammatory state in TCs that may explain the higher immune activation previously reported in ECs [7].

Further evidence observed in our study supporting this hypothesis was the downregulation of complement and coagulation cascade components (carboxipeptidase B2, FA11, and complement component C8 gamma chain) before the loss of spontaneous HIV-1 elite control. This deregulation remained after the loss of virological control, as can be observed with the abundance of 3 complement activation proteins: complement C1r subcomponent, complement factor H, and clusterin compared

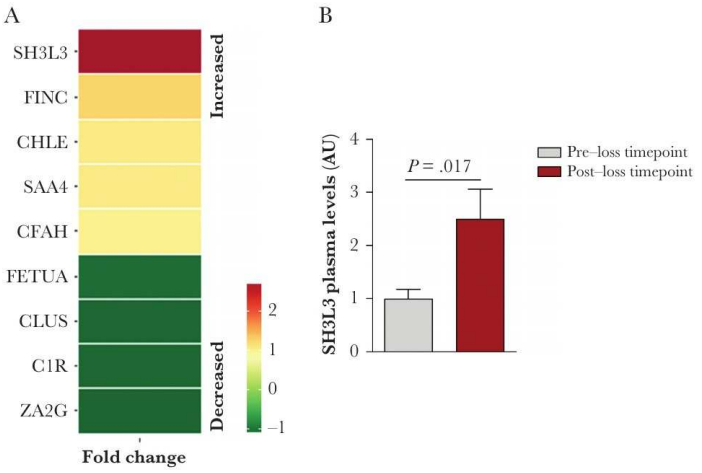


Figure 5. Proteomic analysis comparing transient controllers (TCs) before and after the loss of natural human immunodeficiency virus type 1 control. Heat map representation of the fold change of the differential and significant protein expression as an effect of the loss of control (*A*). The scale from green (low abundance) to red (high abundance) represents the normalized abundance in arbitrary units. Fold change was calculated as A/B – 1, where A was the mean value of –T1 (1 year before loss of virological control) and –T2 (2 years before loss of virological control) and B was the mean of T1 (1 year after the loss of virological control) and T2 (2 years after the loss of virological control). The most remarkable change was the increased levels of SH3 domain-binding glutamic acid rich–like protein 3 after the virological progression in TCs (*B*). Abbreviations: AU, arbitrary units; C1R, complement C1r subcomponent; CFAH, complement factor H; CHLE, cholinesterase; CLUS, clusterin; FETUA, alpha-2-HS-glycoprotein; FINC, fibronectin; SAA4, serum amyloid A-4 protein; SH3L3, SH3 domain-binding glutamic actid rich-like protein 3; ZA2G, zinc-alpha-2-glycoprotein.

with the under-control period in TCs. The role of complement activation proteins in viral pathogenesis has widely been studied in the HIV-1 and HIV/HCV-coinfection scenario [25, 26].

Random Forest analysis, including the combination of the selected proteins, highlighted the importance of FCN2 and 1433Z. Recent findings have demonstrated a protective role of FCN2 against HIV-1 infection, blocking the entry of the virus into the target cells by direct interaction with gp120 [27]. The overexpression of FCN2 found in TCs before the loss of control could be a reflection of partially restricted viral replication despite undetectable VL in TCs. Regarding 1433Z, it plays an important role in cell-cycle checkpoint regulation and has previously been suggested as a potential biomarker for HIV-1–related neurodegeneration [28].

Of special interest is LG3BP, which was the most remarkable biomarker for group discrimination after ROC curve analysis with a concentration that was 2-fold higher in the TC group than PCs. LG3BP is a glycoprotein known to be implicated in macrophage activation, cell signaling, and migration. Regarding the HIV-1 scenario, LG3BP has been associated with increased HIV-1 replication and transmission through a direct interaction with viral *gp120* and host $CD4^+$ T cells [29–31]. This result not only identifies LG3BP as a reliable biomarker for the rapid screening of potential ECs with virological loss of control but also suggests the immunomodulation of this glycoprotein as a therapeutic target in ECs and extensive to HIV remission in the general population.

In this work, a differential proteomic profile in plasma allows us to discriminate 2 EC phenotypes. This finding enhances the recent idea that suggests that HIV-1 controllers might be a heterogeneous group of subjects with different characteristics that remain partially unknown [9, 32]. In addition, our data provide assistance for the design of new therapeutics aiming to achieve sustained virological remission.

The main limitation of this work is the small sample size. However, it has to be highlighted that this type of patient is rare, it is difficult to have this follow-up with stored samples, and this cohort has been used in previous published own works [9, 32]. Despite the relatively low number of participants, our highly sensitive method shows a wide spectrum of proteins (293 proteins were identified and quantified), and the most relevant proteins were confirmed by Western blot. However, further studies using larger cohorts are needed for the establishment of the proposed proteins as biomarkers for the loss of virological control in ECs. Moreover, the lower time of diagnosis observed in TCs may be considered an inherent characteristic of this group because of the faster loss of EC status.

In conclusion, our study determined multiple pathways and deregulated proteins that lead to an inflammatory state that precedes the loss of spontaneous HIV-1 control and which is also maintained after the loss of control. These proteins could be considered potential predictive biomarkers to rapidly screen future loss of natural control, and they provide new clues for a more accurate definition of ECs, which will help in the identification of important determinants for the persistent natural control of viral replication and disease progression.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Immunometabolism is a key factor for the persistent spontaneous elite control of HIV-1 infection

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ABSTRACT

Background: Approximately 25% of elite controllers (ECs) lose their virological control by mechanisms that are only partially known. Recently, immunovirological and proteomic factors have been associated to the loss of spontaneous control. Our aim was to perform a metabolomic approach to identify the underlying mechanistic pathways and potential biomarkers associated with this loss of control.
Methods: Plasma samples from EC who spontaneously lost virological control (Transient Controllers, TC, $n = 8$), at two and one year before the loss of control, were compared with a control group of EC who persistently maintained virological control during the same follow-up period (Persistent Controllers, PC, $n = 8$). The determination of metabolites and plasma lipids was performed by GC-qTOF and LC-qTOF using targeted and untargeted approaches. Metabolite levels were associated with the polyfunctionality of HIV-specific $CD8^+$T-cell response.
Findings: Our data suggest that, before the loss of control, TCs showed a specific circulating metabolomic profile characterized by aerobic glycolytic metabolism, deregulated mitochondrial function, oxidative stress and increased immunological activation. In addition, $CD8^+$ T-cell polyfunctionality was strongly associated with metabolite levels. Finally, valine was the main differentiating factor between TCs and PCs.
Interpretation: All these metabolomic differences should be considered not only as potential biomarkers but also as therapeutic targets in HIV infection.
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1. Introduction

Elite controllers (ECs) are rare individuals who are able to naturally control viral load (VL) below the detection limit in the absence of combined antiretroviral therapy (cART) [1]. It has been suggested that ECs hold the key to how a functional HIV cure can be reached [2]. In-depth characterization of these patients has identified multiple immunological mechanisms involved in the controller phenomenon including host genetic factors, such as HLA-B*57 prevalence [3], a distinct and effective HIV-1-specific T-cell response mediated by high cytokine and chemokine production [4,5] and a remarkably broad array of HLA class II molecules [6].

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[3] The clinical centres and research groups that contribute to ECRIS are shown in the Annex I in Supplementary Data.

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Research in context

Evidence before this study

Elite controllers are those HIV-infected patients who maintain an undetectable viral load without the use of combined antiretroviral treatment. This definition makes them the closest model to the functional HIV cure. However, some studies have elucidated that this group of patient is not homogeneous. Actually, there is a subgroup of elite controllers who eventually loses viral control. The mechanisms that dictate whether an elite controller is going to lose the virological control are still unknown.

Added value of this study

Previous studies reported that differences in metabolite plasma levels are relate to changes in the metabolic flux of T-cells due to its effector functions need significant energetic and biosynthetic requirements. However, these metabolic alterations have not been described in relation to HIV spontaneous control, neither have been associated to immune parameters associated with persistent HIV control. We found for the first time a metabolic reprogramming associated with persistent HIV spontaneous control consisting in increased glycolyis in subjects who were going to transiently control HIV. Importantly this metabolic profiling was associated with HIV-specific CD8 + T-cell response, one of the main factors associated to HIV spontaneous control to date.

Implications of all the available evidence

The current study envisages a specific metabolomic signature associated with the spontaneous loss of virological control in elite controllers. This metabolic profile was characterized by immunometabolism deregulation. All these observed metabolic differences can not only be used as potential biomarkers for a rapid screening of the loss of spontaneous virological control but can also be suggested as susceptible targets for the design of immunotherapeutic strategies in order to achieve the long-term HIV remission without treatment.

Several observations have confirmed that ECs are heterogeneous from a clinical point of view and with regard to both immunological [7–9] and virological features, including a variable proportion of ECs who lose HIV control over time [10,11]. That heterogeneity also reflects that no single mechanism is responsible for controlling viral replication. In fact, recent findings have identified persistent controllers (PCs), compared to subjects who eventually lose viral control, as subjects with low viral diversity, low HIV-DNA levels, decreased immune activation and proinflammatory cytokine levels, efficient high Gag-specific T-cell polyfunctionality and a proteomic profile characterized by lower levels of inflammation, transendothelial migration and coagulation [12–15]. PCs may help to define the right model of functional cure and may be a good example for the design of eradication strategies and HIV vaccine development.

New studies of immune system metabolism ("immunometabolism") have tried to explore the role of metabolic pathways within immune cells and how this interplay regulates immune response outcomes. It is becoming apparent that changes in the metabolomic profile of cells upon viral infection are important for viral replication and importantly for the seeding of HIV reservoir in $CD4^+$ T-cells [16–18]. The measurement of specific metabolic products in plasma has revealed differences in the phenotypes of HIV-infected individuals and identified biomarkers associated with HIV natural evolution [19–21] that may participate in viral containment together with distinct immune features. While not much is known in HIV-1 controllers, a distinct tryptophan catabolic feature may explain the persistence of the functional T-cell response [22,23]. However, to date, no longitudinal study determining the metabolomic profile associated with the loss of spontaneous HIV-1 elite control has been performed, and there is no evidence of an interplay between the metabolic signature and immune outcomes in these patients.

Given the importance of immunometabolism in HIV-1 infection and the lack of metabolomic information in this heterogeneous group of HIV-infected patients, the performance of metabolomic approaches could reveal important metabolic differences between persistent and transient elite controllers, which could lead to new insights into future designs of effective vaccines and cure strategies using persistent spontaneous control as a model. Therefore, the purpose of this study was to perform targeted and untargeted metabolomic strategies in order to study the underlying mechanisms associated with the loss of spontaneous virological control in ECs and to identify potential predictive biomarkers that could explain the inefficient HIV-specific T-cell response observed in transient controllers.

2. Methods

2.1. Patients and study design

ECs were defined as subjects with VL determinations below the detection limit (<50 HIV-1-RNA copies/mL) in the absence of cART for at least 12 months. A total of 16 subjects were included in the study based on frozen plasma and peripheral blood mononuclear cell (PBMC) sample availability according to a similar study design that was previously described [12,13] (Fig. 1). The samples were received, processed and stored in the Spanish HIV HGM BioBank belonging to the AIDS Research Network (RIS) [24], and data were registered in the RIS cohort of HIV Controllers Study Group (ECRIS) (Annex I, Supplementary Material). A detailed description of the cohort's characteristics has been previously published [11,25]. The 16 subjects were analysed; eight ECs who experienced loss of spontaneous virological HIV-1 control (at least two consecutive measurements of VL above the detection limit in 12 months) were called transient controllers "TCs", and another group of eight ECs who persistently maintained virological control during the same follow-up period were called persistent controllers "PCs" (see the study design in Fig. 1). Up to four determinations were assessed in TCs: at two years and one year before the loss of virological control, "pre-loss of control period", (−T2 and −T1 respectively), and two determinations in the "post-loss of control period", including the closest time point and one year after the loss of virological control (+T1 and + T2, respectively). In PCs, up to two determinations were assessed at one-year intervals (+T1 and +T2).

None of the subjects took drugs with known metabolic effects (such as lipid-lowering agents or antidiabetics agents, among others). As an example, it has been well described the immunomudulatory and

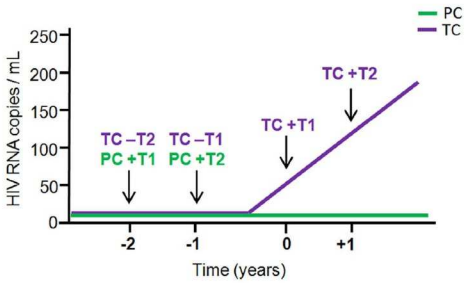


Fig. 1. Schematic representation of the study design and follow-up time points determined in transient controllers (TCs) and in persistent controllers (PCs).

anti-inflammatory effects of statins in infectious disease [26].Therefore, its use may bias immune response and lipidomics results. For this reason, its use was considered within the exclusion criteria. All subjects included in the study gave their informed consent, and protocols were approved by the institutional ethical committees.

Laboratory evaluations were performed at the Laboratory of Joan XXIII University Hospital in Tarragona, IISPV, Rovira i Virgili University (Spain) and at the Laboratory of Immunovirology, Institute of Biomedicine of Seville (IBiS), Virgen del Rocío University Hospital in Seville (Spain).

2.2. Laboratory determinations

Absolute counts of $CD4^+$ and $CD8^+$ T-cells were determined in fresh whole blood by using an Epic XL-MCL flow cytometer (Beckman-Coulter, Brea, California) according to the manufacturers' instructions. Plasma HIV-1 RNA concentration was measured by quantitative polymerase chain reaction (COBAS Ampliprep/COBAS Taqman HIV-1 test, Roche Molecular Systems, Basel, Switzerland) according to the manufacturer's protocol. The detection limit for this assay was 50 HIV-1-RNA copies/mL. Hepatitis C virus (HCV) RNA was determined using an available PCR procedure Kit (COBAS Amplicor, Roche Diagnosis, Barcelona, Spain) with a detection limit of 10 IU/mL.

Total DNA was extracted from peripheral blood mononuclear cells (PBMCs) using the MagNa Pure LC DNA isolation kit. HLA-B group alleles were genotyped using a reverse sequence-specific oligonucleotide bound to a fluorescently coded microsphere system (LABType SSO, RSSO1B, One Lambda, Canoga Park, CA), following manufacturer's instructions.

2.3. Determination of the Krebs cycle metabolites and energy metabolism by GC-(EI)qTOF/MS

Metabolite extraction from plasma samples was performed by a protein precipitation method with methanol/water 8:1 (v/v) containing an internal standard mixture. Supernatants were collected and evaporated to dryness under N_2 stream and freeze dried before derivatization with methoxyamine hydrochloride and MSTFA +1% TMCS. Samples were analysed on a 7200 GC-QTOF Gas chromatograph-quadrupole time of flight mass spectrometer (GC-qTOF) from Agilent Technologies (Sta. Clara, CA, USA) using a HP5-MS chromatographic column from J&W Scientific (30 m × 0·25 mm i.d., 0·25 μm film) and helium (99·999% purity) as a carrier gas. Ionization was performed by electronic impact (EI), with an electron energy of 70 eV, and the mass analyzer was operated in Full Scan mode, recording data in a range between 35 and 700 *m/z* at a scan rate of 5 spec/s [27,28].

Absolute quantification of the Krebs cycle metabolites was performed with an internal standard calibration curve using the corresponding analytical standard for each determined metabolite and a deuterated internal standard depending on the family of metabolite. In addition, an untargeted approach was performed by deconvoluting the acquired raw data by Unknown Analysis software from Agilent and using Fiehn RT library to identify by EI-MS spectra and library retention time the compounds present in the sample. For these compounds, their peak areas were used for relative quantification between samples.

2.4. Liposcale test

We measured triglyceride and cholesterol concentrations, the size and particle numbers for very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) classes, and the particle numbers of nine subclasses: large, medium and small VLDLs, LDLs and HDLs of frozen samples using the Liposcale test as described elsewhere [29–31].

2.5. Lipidomic analysis by LC-qTOF/MS

Lipid extraction from plasma samples was carried out by a liquid-liquid extraction using methyl-tert-butyl ether/methanol/water. Then, the upper organic layer was collected, evaporated to dryness in a SpeedVac concentrator and reconstituted with methanol:toluene (9:1, v,v) for analysis by UHPLC-(ESI)qTOF.

Samples were analysed on an Agilent 1290 Infinity UHPLC coupled to an Agilent 6550 qTOF mass spectrometer. The chromatographic column used was a Kinetex C18-EVO (2·1 × 100 mm, 2 · 7 μm) from Phenomenex, and the mobile phase was 2-propanol/methanol/water (5:4:1) and 2-propanol/water (99:1), both with 5 mM NH4OAc and 0·1% acetic acid. The mass spectrometry analysis was performed both in positive and negative ionization in separated runs operating in full scan mode between 300 and 1200 *m/z* at 2 spectra/s. Additionally, tandem mass analysis (MS/MS) was performed at a fixed collision energy of 20 eV for a data dependence analysis (DDA) using the 10 most abundant precursor ions with a dynamic exclusion of 30 s for lipid identification.

Data analysis was performed using Mass Profinder software from Agilent Technologies for raw data signal deconvolution using recursive feature extraction algorithm for these features previously identified with SimLipid software (PREMIER Biosoft), ((http://www.lipidmaps.org/). The generated features were aligned across all samples to create a compound matrix to perform statistical analyses.

2.6. Cell stimulation and intracellular cytokine staining

The stimulation assay and intracellular cytokine staining analysis were evaluated previously in a study with a different objective [12].

Briefly, PBMCs were thawed, washed and *in vitro* stimulated with 2 μg/mL of overlapped HIV (Gag)-specific peptide pool (NIH AIDS Research and Referenced Reagent Program (https:// www.aidsreagent.org/index.cfm) and stained with conjugated monoclonal anti-CD107a-BV786 (clone H4A3; BD Biosciences, Franklin Lakes, NJ) at the beginning of incubation as previously described [4,12].

Stimulated PBMCs were washed and stained with LIVE/DEAD fixable Violet Dead Cell Stain (Life Technologies, CA, USA). Then, cells were surface stained with anti-CD14-PB, anti-CD19-PB, anti-CD56-PB (Biolegend, San Diego, CA), anti-CD8+-PerCP-Cy5.5, anti-CD45RA-FITC, anti-CD27-BV605 and anti-CD57-PE-CF595 (BD Biosciences). Cells were then stained intracellularly for 30 min with 100 μl of PBS with anti-CD3-APC-H7, anti-IFN-γ-PCy7, anti-TNF-α-Alexa700, anti-IL-2-APC and anti-Perforin-PE (BD Biosciences) and then washed twice and fixed in PBS containing 4% paraformaldehyde. Unstimulated condition and cell stimulation with staphylococcal enterotoxin B (SEB) condition as a positive control were included in each experiment. Alive lymphocytes were defined as low forward/side scatter, no violet dead cell staining and expressed CD3, and/or no CD8+ but not CD19, CD14, and CD56 (Supplementary Fig. 1). PBMCs were analysed by using an LSR Fortessa Cell Analyzer (BD Biosciences, Spain). A minimum of 1,500,000 total events were recorded for each panel and condition.

2.7. Statistical analysis

All variables were considered non-parametric due to the sample size. Differences between categorical values were determined by the Chi-square test. Differences between unpaired groups were determined by univariate comparisons through the non-parametric Mann-Whitney *U* test. Correlations between variables were assessed using the Spearman rank test. p values $<0·05$ were considered statistically significant. Additionally, the fold change (FC) of each variable was calculated as 'A/B', where 'A' was the median value of the TC group pre-loss time points, and 'B' was median value of the PC group. Alternatively, 'A' was the median value of the post-loss time points in TCs, and 'B'was the

median value of pre-loss time points in TCs. The results were represented with heat maps and hierarchical clustering analysis (HCA).

In PCs, a PCA analysis and Wilcoxon signed-rank test were applied in paired samples to assess metabolite changes as an effect of time. As shown in Supplementary Fig. 2, no changes were observed over time in PCs. Thus, we simplified the following analysis to a single variable, which was expressed as the mean value of the two consecutive longitudinal determinations in PCs. We then focused the analysis on pre-loss of control time points from TCs compared to PCs in order to identify potential biomarkers for the loss of spontaneous HIV-1 control. To simplify, for all the comparisons, we confirmed that no differences were observed between –T1 and –T2, and +T1 and +T2 (data not shown), and the mean values of the –T1 and –T2 time points (pre-loss time point) and +T1 and +T2 time points (post-loss time point) were calculated.

Multivariate statistics were also used to improve the refining and distilling of all the metabolomics data and for pattern recognition purposes. Thus, principal component analysis (PCA) was performed. Moreover, random forest analysis receiver operating characteristic (ROC) curves were also generated to identify the variables that make the largest contributions to the discrimination between groups.

Polyfunctionality was defined as the percentage of lymphocytes producing multiple cytokines. Polyfunctionality was quantified with the polyfunctionality index algorithm [32] employing the 0.1.2 beta version of the "FunkyCells Boolean Dataminer" software (www.FunkyCells.com) provided by Dr. Martin Larson (INSERM U1135, Paris, France).

The statistical software used included the programme 'R', version 3.4.4 (http://cran.r-project.org) and the SPSS 23.0 package (IBM, Madrid, Spain). Graphs were generated with Prism, version 5.0 (GraphPad Software, Inc.).

3. Results

3.1. Characteristics of the studied subjects

Subjects were recruited based on samples availability based on the study design shown in Fig. 1. The clinical and demographic characteristics of the subjects at baseline (first follow-up time point) were defined. As shown in Table 1, no differences were observed in age, sex, transmission route, HCV coinfection, $CD4^+$ and $CD8^+$ T-cell counts and the CD4:CD8 ratio between TCs and PCs. The TC group presented a shorter time since diagnosis than the PC group (median [interquartile range] of 3 [2–8] *vs* 13 [10–17] years; $p = 0{\cdot}005$). The VL evolution from TCs after the loss of control was 539 [295–1120] at +T1 and 2740 [985–22,250] HIV-RNA copies/mL at +T2. No differences were found in HLA-B57, 27 and 35 frequencies between groups ($p > 0.05$).

Table 1
Patients' characteristics.

	Transient controllers	Persistent controllers	p-Value
	TC (n = 8)	PC (n = 8)	
Age (years)	41 [34–60]	44 [40–46]	0·635
Male sex, n (%)	5 (62.5)	4 (50)	0·614
IDU, n (%)	3 (37.5)	4 (50)	0·198
Time since diagnosis (years)	3 [2–8]	13 [10–17]	**0·005**
HCV RNA detected, n (%)	3 (37·5)	3 (37·5)	0·999
CD4+ T-cells (cell/μL)	676 [623–963]	724 [609–985]	0·817
CD8+ T-cells (cell/μL)	787 [553–1162]	636 [432–1026]	0·482
CD4:CD8 Ratio	0.86 [0·53–1·55]	1.08 [0·93–1·47]	0·406

Values from TC are taken from –T2 and values from PC are taken from the first follow-up time point. Values are given as percentage for categorical variables or median and interquartile range for continous variables. The Mann-Whitney U and Chi-squared tests were used. All p values <0·05 were considered significant and are highlighted in bold. IDU, Intravenous drug users.

3.2. A specific metabolomic signature precedes the loss of spontaneous HIV-1 control in transient controllers

A total of 70 metabolites were identified and quantified by GC-QTOF/MS in plasma samples. When we focused the analysis on pre-loss of control time from TCs compared to PCs in order to identify the metabolomic signature associated with the loss of spontaneous HIV-1 control, only the plasma levels of thirteen metabolites were significantly different (Fig. 2A). Fig. 2B shows the data distribution of some of these 13 significantly metabolites. In addition, these 13 metabolites showed good clustering and reliable differentiation between the two studied groups (Fig. 2C–D).

A Random Forest analysis (out-of-bag (OOB) error = 12·5%) was then performed in order to select the best parameter for group discrimination. As shown in Fig. 2E, valine, iminodiacetic acid, 2-ketoisocaproic acid, pyruvic acid, alpha tocopherol, succinic acid and glycolic acid showed higher classification power (mean decreased accuracy (MDA) > 50). Finally, receiver operating characteristic (ROC) curves demonstrated that, among the 13 significant metabolites, valine was the only metabolite that could discriminate TCs from PCs with 100% sensitivity and specificity (Fig. 2F).

A metabolic pathway analysis revealed that the most significant deregulated metabolites were mainly involved in glycolysis, the Krebs cycle and amino acid metabolism. A schematic representation including the statistically significant metabolites between groups and the rest of metabolites involved in the pathway are shown in Fig. 3.

Finally, regarding changes in TC metabolites as an effect of detectable viremia, only monostearin levels were different between TCs at pre-loss and post-loss time points, being significantly lower after the loss of control (Supplementary Fig. 3).

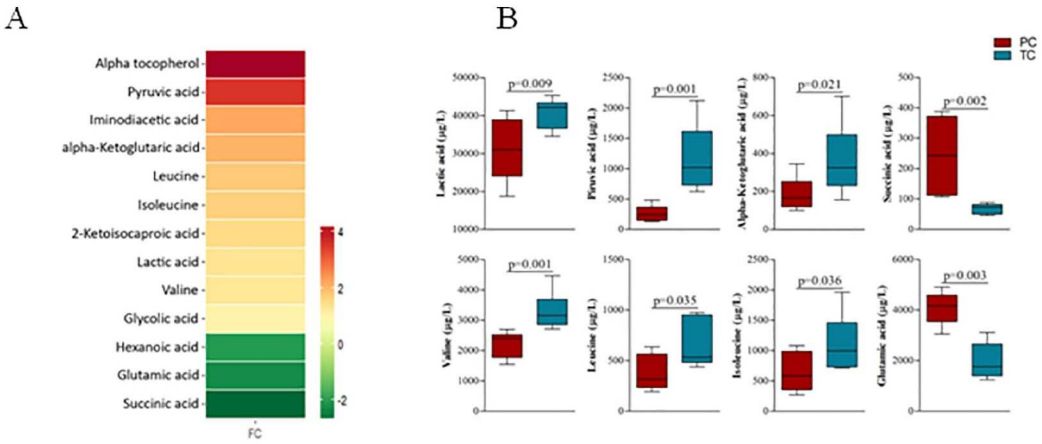
3.3. Gag-specific $CD8^+$ T-cells response is associated with metabolite levels

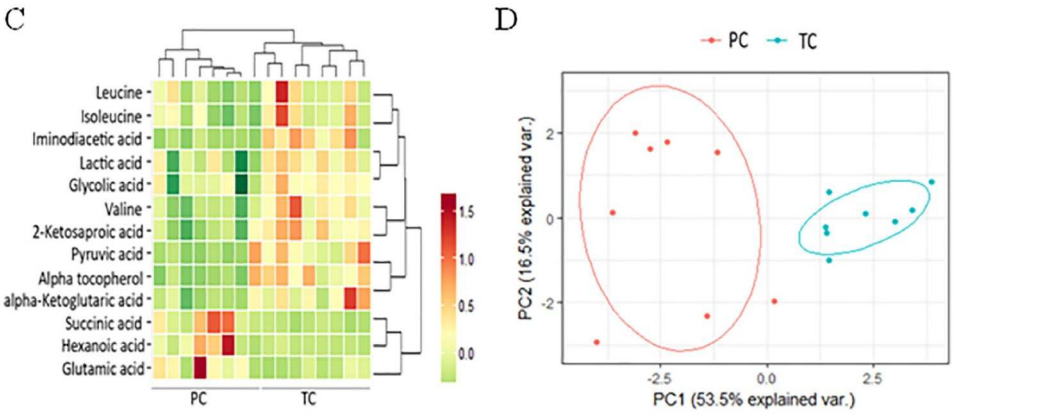
In a previous work [12], we showed that PCs presented higher levels of Gag-specific CD4+ and $CD8^+$ T-cells than TCs before the loss of control, as determined by the intracellular production of IFN-γ, TNF-α and IL-2 by T cells. Polyfunctionality, which is understood as simultaneous multiple production of these cytokines by T cells, was studied only in subjects categorized as responders and calculated by the index of polyfunctionality (pINDEX). T-cell response was defined as the frequency of cells (>0·05%) with detectable IFN-γ, TNF-α and/or IL-2 intracellular cytokine production after stimulation. Due to the low number of Gag-specific $CD4^+$ T-cell responders among TCs, polyfunctionality was only assessed in responders with $CD8^+$ T-cells.

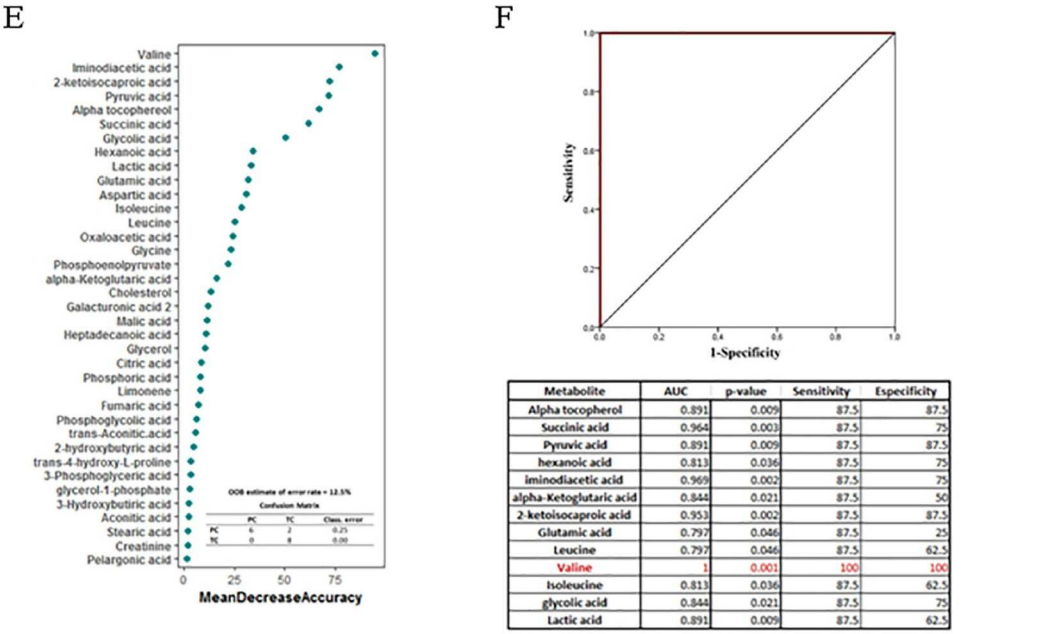
To associate immunological changes with the most significant metabolic results, three-function CD8+ T-cell polyfunctionality (calculated by the pINDEX) from PCs and TCs before the loss of control was strongly inversely correlated with alpha tocopherol, pyruvic acid, alpha-ketoglutaric acid, and 2-ketoisocaproic acid plasma levels ($r = -0{\cdot}82$, $p = 0{\cdot}001$; $r = -0{\cdot}78$, $p = 0{\cdot}003$; $r = -0{\cdot}61$, $p = 0{\cdot}035$; and $r = -0{\cdot}618$, $p = 0{\cdot}032$, respectively) and directly associated with hexanoic acid ($r = 0{\cdot}69$, $p = 0{\cdot}012$) (Fig. 4A–E). The same associations with those metabolites remained significant with 4- and 5-function total CD8+ T-cell polyfunctionality (cytokine combinations plus CD107a and plus perforin, respectively; data not shown).

3.4. Plasma lipid analysis comparing persistent controllers and transient controllers and changes in transient controllers due to the loss of control

Using the Liposcale test we found no significant differences in triglycerides, cholesterol and lipoproteins comparing PCs and TCs before the loss of control. Regarding paired samples from TCs, LDL-cholesterol, the number of medium LDL particles and non-HDL particles and the ratio of LDL particles/HDL particles were increased after the loss of control ($p = 0{\cdot}039$, $p = 0{\cdot}007$, $p = 0{\cdot}039$ and $p = 0{\cdot}015$ respectively).







Metabolite	AUC	p-value	Sensitivity	Especificity
Alpha tocopherol	0.891	0.009	87.5	87.5
Succinic acid	0.964	0.003	87.5	75
Pyruvic acid	0.891	0.009	87.5	87.5
hexanoic acid	0.813	0.036	87.5	75
iminodiacetic acid	0.969	0.002	87.5	75
alpha-Ketoglutaric acid	0.844	0.021	87.5	50
2-ketoisocaproic acid	0.953	0.002	87.5	87.5
Glutamic acid	0.797	0.046	87.5	25
Leucine	0.797	0.046	87.5	62.5
Valine	1	0.001	100	100
Isoleucine	0.813	0.036	87.5	62.5
glycolic acid	0.844	0.021	87.5	75
Lactic acid	0.891	0.009	87.5	62.5

On the other hand, similar to the metabolomic approach described before, we also analysed differences in the plasma lipidome from PCs and TCs before the loss of control. A total of 334 lipids were detected in plasma, but only 93 were significantly different between the two groups (Supplementary Table 1). We then ordered these significant lipids according to their classification power using a Random Forest analysis (OOB error = 6·25%). The results demonstrated that only 7 lipids performed with an MDA higher than 50 (Fig. 5A). These plasma lipid levels were significantly lower in TCs before the loss of control than in PCs, but, more importantly, as shown by the PCA analysis (Fig. 5B), these lipids had great discriminatory potential between the studied groups.

Regarding changes in TC plasma lipids as an effect of viral loss of control, 43 lipids were significantly different after the loss of control compared with pre-loss time points in TCs; most of them decreased after the loss of virological control (Supplementary Fig. 4).

3.5. Lipid levels are associated with Gag-specific CD8$^+$ T-cell response and metabolite plasma levels

To identify the associations between lipids and Gag-specific CD8 + T-cell response, we focused on the most significant lipids associated with the loss of control mentioned before. All of these plasma lipid levels were higher in PCs than in TCs before the loss of control, and interestingly, these levels were strongly and directly associated with the three-function CD8+ T-cell pINDEX ($n = 11$) ($p < 0·001$ for all associations) (Fig. 6A–G). The same associations with these lipids remained significant with 4- and 5-function total CD8+ T-cell polyfunctionality (plus CD107a and plus perforin, respectively) (Supplementary Fig. 5 A–H).

Correlations between the most relevant lipids and metabolites associated with the loss of control are represented in Supplementary Fig. 6. Lipid and metabolite levels were strongly associated in PCs and TCs before the loss of control ($n = 15$).

4. Discussion

The current study elucidated a metabolomic signature associated with the spontaneous persistent elite control of HIV-1 infection and that distinguish the transient controllers from persistent controllers. There were strong interrelations among metabolites, lipids levels and an important immune function parameter associated with spontaneous control of HIV as is HIV-specific T-cell response. Moreover, the amino acid valine stood out as the main differentiating factor between the studied groups.

Among the observed metabolomic changes, the most significant differences were related to metabolic pathways as important as glycolysis, the Krebs cycle, and amino acid catabolism, mostly that of branched chain amino acids. Previous studies have demonstrated that all these pathways are targeted during viral infection [16,33], but so far these metabolic disturbances in energy metabolism have not been described in HIV-1 elite controllers.

Immunometabolism is now an important field in immunology. Microbial infections results in important changes to the physiology and metabolism of innate immunity host cells, such as macrophages and T-cells. In this sense, differences in metabolite plasma levels may reflect changes in cellular metabolic flux and could be also related to the outcome of the infectious process [34,35].

T-cell activation, T-cell mediated antiviral responses and other T-cell effector functions require significant energetic and biosynthetic requirements from the cell [36,37]. During HIV infection, energy demand increases dramatically, which is translated as a decrease in circulating glucose levels (the main cellular energy source) and activation of the glycolysis pathway [37,38]. This is exactly what we observed in TCs before the loss of control: a lower glucose concentration and a significant increase in glycolytic intermediates, such as 3P-glycerate, phosphoenolpyruvate and pyruvate, compared with PCs. Some studies have proposed that the accumulation of glycolytic intermediates is used in activated T-cells for biosynthetic processes [39]. Moreover, our previous results demonstrated that TCs are characterized by high immune activation and a distinct inflammatory profile [12,13], which can be explained by the observed metabolomic differences. Recent studies have also highlighted the role of glycolytic enzymes and intermediates in regulating T-cell cytokine production, especially IFN-γ, which reinforces our results [40,41]. Moreover, regarding HIV-1 infection, glucose metabolism provides precursors for HIV biosynthesis and intermediate enzymes that may disengage glycolysis and regulate HIV reactivation [42]. Importantly, a recent study has highlighted the important link between increased glycolysis and HIV-1 reservoir seeding in CD4$^+$ T-cells [17]. This is in accordance with our data in TC who also showed higher proviral DNA levels [12].

Furthermore, activated T cells undergo a metabolic reprogramming; instead of relying on mitochondrial oxidative phosphorylation to generate the energy needed for cellular process, they enhance aerobic glycolysis, a phenomenon commonly known as the Warburg effect [43]. As a result, activated lymphocytes increase lactate production even in the presence of oxygen [44,45]. This is exactly what we observed in TCs at pre-loss time points.

Other studies have proposed lactic acidemia or hyperlactatemia as an indicator of mitochondrial oxidative deregulation [46–48]. In this sense, our results showed a general deregulation of the Krebs cycle metabolites in TCs before the loss of control. Actually, only the increased Krebs cycle intermediates in TCs before the loss of control compared to PCs are those that can be supplied by anaplerotic reactions. The most remarkable example is α-ketoglutarate concentration, which was increased in TCs. This could be directly related to the significant decrease in glutamate levels. When energy levels are reduced, glutamate can be converted into α-ketoglutarate and can enter the Krebs cycle for energy production, trying to fully compensate for the lack of oxidative Krebs cycle activity. Moreover, another indicator of defective mitochondrial function was the significant decrease in succinate levels in TCs [49–51], reinforcing the key role of mitochondria in the progression of HIV infection [52,53].

As mentioned before, our results suggested that valine is the main differentiating factor between PCs and TCs. Valine is one of a group of the three essential amino acids with crucial roles in metabolism, called branched-chain amino acids (BCAAs: leucine, isoleucine and valine) [54]. Our results demonstrated a significant increase in this type of amino acids and in some of their catabolic intermediates, such as α-ketoisocaproic acid, in TCs before the loss of control compared to PCs. In this sense, some previous studies have linked high concentrations of BCAAs with deficiencies in its catabolism [55]. Thus, since the catabolic products of BCAAs could enter the Krebs cycle for energy generation, the accumulation of these products may also be related to activated T-cell reprogramming into glycolytic metabolism, reducing the oxidative phosphorylation pathways [43,56,57].

Fig. 2. Metabolomic analysis comparing PCs and TCs before the loss of natural HIV-1 control. Heat map representation of the fold change of each statistically significant metabolite, the scale from green (low abundance) to red (high abundance) represents the normalized abundance in arbitrary unit (A). Box-plots graph of some of the 13 significantly metabolites Hierarchical combined tree showing the clusterization of metabolites (C) and Principal Component Analysis (PCA) showing that these 13 metabolites allow the differentiation between the studied groups (D), TCs (blue, $n = 8$) and PCs (red, n = 8). Random Forest analysis showed metabolites by importance of classification (E). Logistic regression and receiver operator characteristic (ROC) curves elucidated the amino acid valine as the main differentiator between PCs and TCs before the loss of control (F).

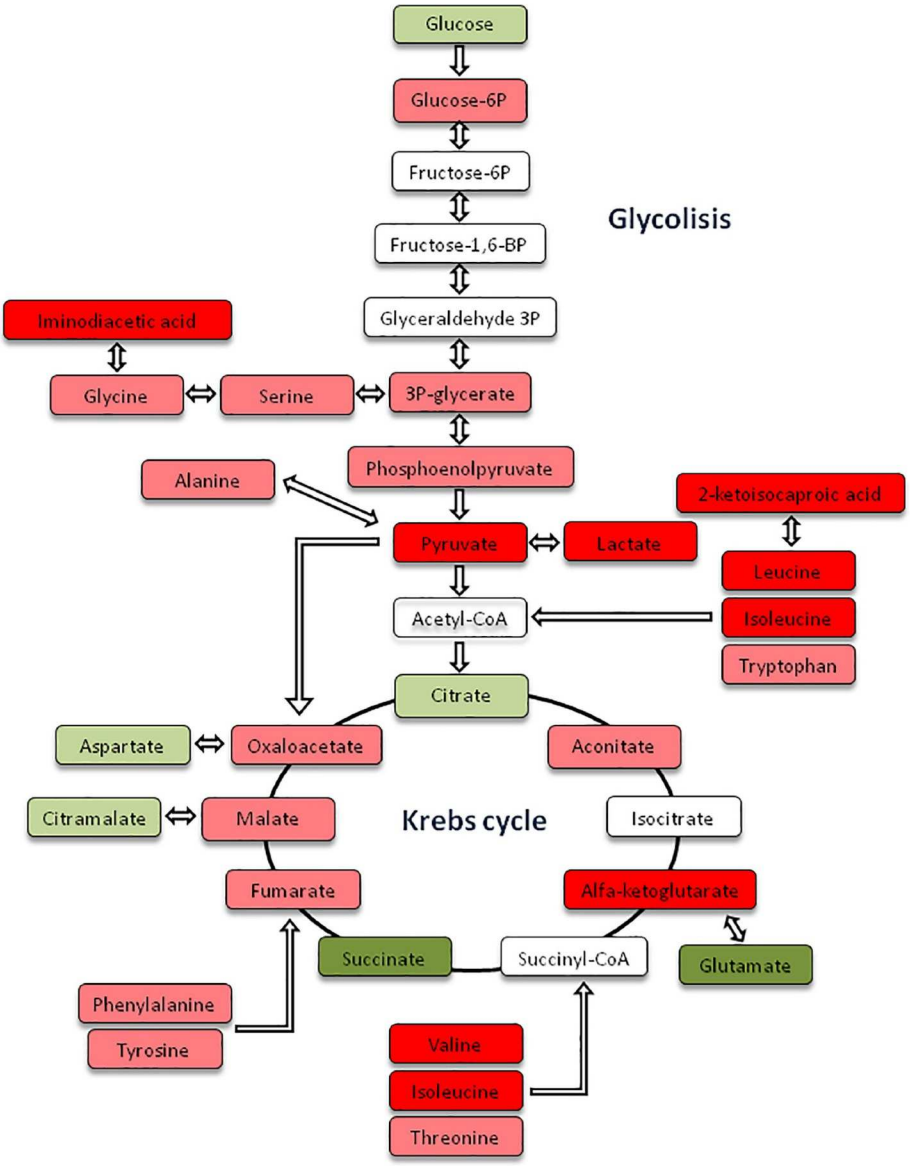


Fig. 3. A schematic representation of the relevant deregulated metabolic pathways involved in the loss of virological control. Metabolites observed at lower levels in TCs compared with PC are shown in green (dark green $p < 0{\cdot}05$, light green $p > 0{\cdot}05$); increased levels of metabolites observed in TCs are shown in red (dark red $p < 0{\cdot}05$, light red $p > 0{\cdot}05$); and undetected metabolites are shown in white.

High concentrations of BCAAs also promote oxidative stress and, therefore, mitochondrial damage and dysfunction [58]. α-tocopherol, commonly known as vitamin E, plays an important role as an antioxidant agent and has been used in HIV-infected patients as a supplement to reduce oxidative stress [59]. Our results demonstrated that TCs have higher levels of vitamin E that may mirror a compensatory mechanism.

The changes in mitochondrial dynamics in TCs presented herein also explain the differences observed in lipid levels. Mitochondria have an important role in the maintenance of lipid homeostasis by orchestrating the synthesis of key membrane phospholipids [60]. Moreover, phospholipids are the main building blocks of cell membranes; thus, changes in phospholipid composition could affect mitochondrial function, structure and biogenesis and rely on the metabolism of phospholipids themselves [61]. Therefore, it is not surprising that phospholipid alterations have been associated with several diseases, including HIV infection [61,62]. During infection, innate immune cells suffer from significant remodelling of their lipid compartments, including the plasma membrane [63]. Moreover, lipid synthesis is critical in effector T-cell differentiation [64] and secondary responses of memory $CD8^+$ T-cells [65]. Interestingly, in Pernas et al., we observed that the effective Gag-specific response from PCs was mediated mainly by the central memory $CD8^+$ T-cell subset [12]. This may explain the associations between lipid plasma levels and Gag-specific response that we have shown in this work. The specific role of each lipid subclasses on immune cells is uncertain and may vary according to lipid subtypes, but

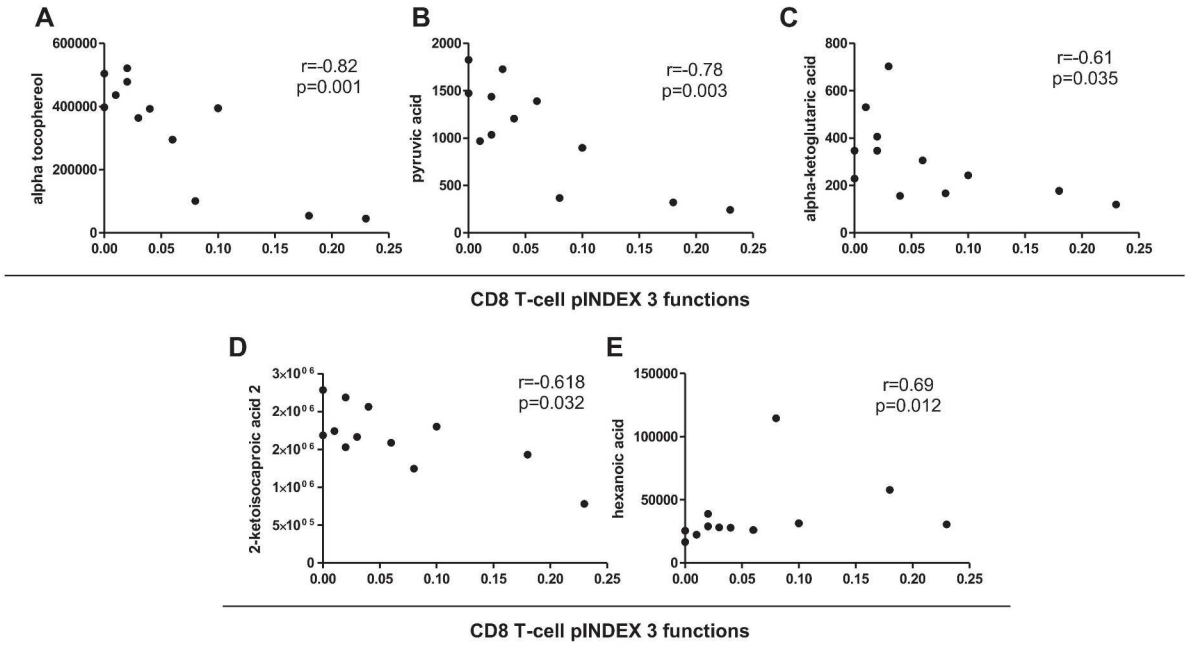


Fig. 4. Correlation of plasma levels of metabolites with CD8+ T-cell polyfunctionality. Correlations between alpha tocopherol (A), pyruvic acid (B), alpha-ketoglutaric acid (C), 2-ketoisocaproic acid 2 (D), hexanoic acid (E) levels and Gag-specific total CD8+ T-cells expressing combinations of IFN-γ, TNF-α, IL-2 (pINDEX 3-functions) from PCs and TCs before the loss of virological control ($n = 12$ in each analysis). The Spearman ρ correlation coefficient test was used.

new evidence is emerging in the scenario of HIV infection [66–68]. Modulation of fatty acid synthesis and oxidation pathways could be used as strategies to prevent inflammatory T-cell differentiation.

The main limitation of our study is the small sample size of our study population. However, as mentioned in previous our published works using this cohort [12,13], these types of patients are rare, and it is difficult to perform a long follow-up with stored available samples. For this reason and to try to overcome this constraint, we have used a highly sensitive mass spectrometry approach. Moreover, the shorter time for diagnosis observed in TCs may be considered an inherent characteristic of this group because of the faster loss of EC status.

In conclusion, this study envisages a specific metabolomic profile associated with the spontaneous loss of virological control in ECs. This profile was characterized by T-cell metabolic reprogramming to the aerobic glycolytic pathway and by a decrease in mitochondrial function and increased oxidative stress and immunological activation. Metabolite and lipid plasma levels were also strongly correlated with immunological parameters. Moreover, valine could be considered a potential biomarker for the prediction of virological progression in elite controllers. Therefore, all these observed metabolic differences can not only be used as biomarkers for rapid screening of future loss of spontaneous control but can also be suggested as therapeutic targets in HIV-1

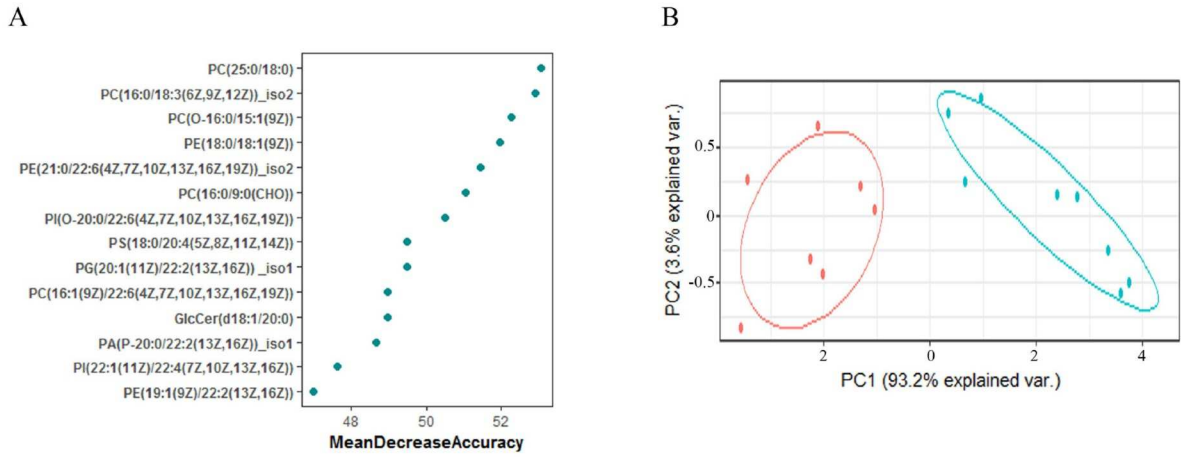


Fig. 5. Lipidomic analysis comparing TCs before the loss of HIV-1 control and PCs. Random Forest analysis showed lipid levels by importance of classification (A). Principal Component Analysis (PCA) showing that lipids with a mean decrease in accuracy higher than 50 allowed a great differentiation between the studied groups (B). Transient controllers (TCs, blue, n = 8) and persistent controllers, (PCs, red, n = 8).

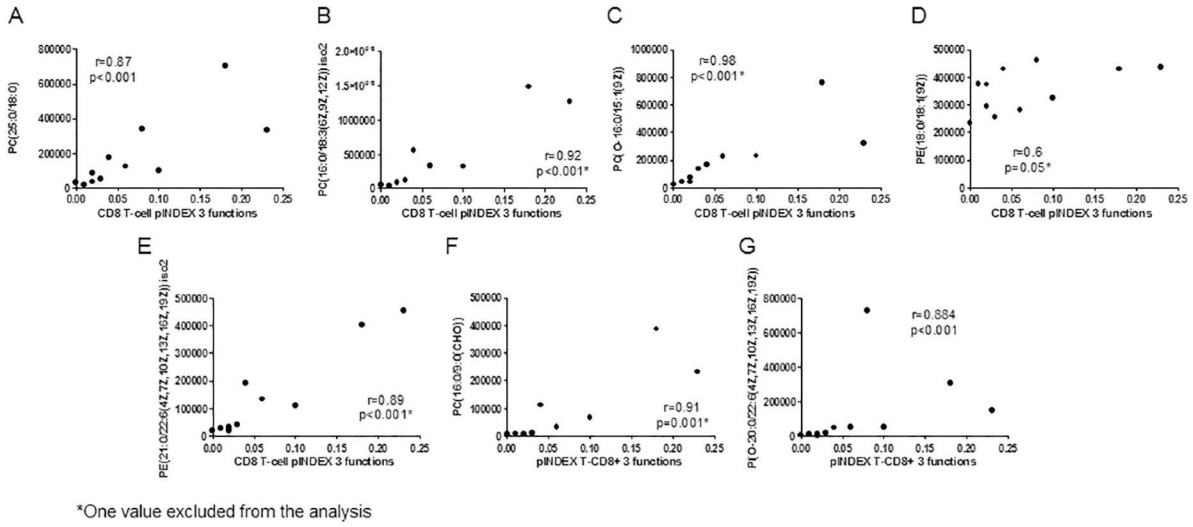


Fig. 6. PC(25:0/18:0), PC(16:0/18:3(6Z,9Z,12Z)) iso2, PC(O-16:0/15:1(9Z)) and PE(21:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) iso2 plasma levels correlations with Gag-specific CD8+ T-cell pINDEX 3-function from PCs and TCs before the loss of control ($n = 11$). An outlier (*) excluded from the analysis ($n = 10$). The Spearman ρ correlation coefficient test was used.

infection. New strategies focused on improving metabolic and inflammatory conditions in HIV-1 patients are needed in order to enhance protective immunity. In this sense, the use of statin and aspirin are being use in clinical trials (NCT02081638), and in addition antioxidant vitamins, amino acids or other dietary supplements have started to be widespread in the HIV-infected community [18]. Based on our results, metformin and thiazolidinediones, widely used to treat type 2 diabetes, could be potential therapies given that it has been demonstrated that they improve glucose metabolism [69]. Another therapeutic option could be the use of antioxidants, such us vitamin E mentioned before [59]. Moreover, some studies are evaluating the potential benefit of niacin or vitamin B3 in reducing immune activation, another important point during HIV-1 infection [70]. However, further external validation studies with larger cohorts are needed to consolidate our results in order to identify the persistent controller phenotype and to pinpoint susceptible targets for the design of immunotherapeutic strategies with the aim of achieving long-term HIV remission in the absence of ART.

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Declaration of interests

The authors declare no competing interests.

Author contributions

FV and ER-M conceived and coordinated the study development. LT-D, ER-G and AR designed and performed experiments, analysed and interpreted the data, designed the figures and wrote the manuscript. VA and PH contributed in sample preparation and in mass spectrometry analysis. MR-JL participated in the methodology and data analysis. JP, CV, IP and ML collaborated with patient's characterization and sample collection. ER-M and FV participated in critical revisions for intellectual content. All authors critically reviewed, edited and approved the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.03.004.

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DISCUSIÓN GLOBAL

En conjunto la presente Tesis Doctoral profundiza en el estudio de parámetros inmunológicos y potenciales mecanismos asociados a la inmunopatogenia de la infección por VIH en la era actual representado con la aparición de ECAs y en el control espontáneo de la misma en el escenario del paciente controlador de élite.

Las ECAs son, tras la enfermedad hepática, una de las principales causas de morbimortalidad en la población VIH actualmente (51). Sin embargo, tras la reciente incorporación de los fármacos de acción directa frente al VHC (52) es probable que la prevalencia de ECAs aumente en los próximos años una vez erradicada la infección por VHC y que se convierta en uno de los principales problemas en el manejo clínico de los pacientes VIH bajo TARc. A pesar de que tradicionalmente la ECA se ha asociado a los factores tradicionales de riesgo cardiovascular y al efecto de algunas terapias antirretrovirales (18, 53), hoy en día se conoce que la inflamación y activación del sistema inmunitario también juegan un papel relevante en estos procesos. En esta Tesis Doctoral hemos corroborado y profundizado en esas observaciones identificando un perfil de inflamación crónica sistémico implicado en la aparición de síndrome coronario agudo en pacientes bajo TARc.

Puesto que el VIH per sé se considera uno de los factores responsables del estado de inflamación sostenida del sistema inmunitario (5), no es de extrañar que otras infecciones víricas o bacterianas tengan un efecto acumulativo también en la generación de este estado de activación crónica como hemos identificado también en nuestros trabajos. Estas observaciones son de gran importancia puesto que nos sugieren que tanto el tratamiento frente a coinfecciones como el VHC como la inmunización frente a infecciones víricas y bacterianas (virus herpes zoster, influenza y pneumococco) deben tenerse en cuenta

como estrategia preventiva en el desarrollo de ECAs en la práctica clínica.

El conjunto de nuestras observaciones indican una asociación directa entre las coinfecciones y los niveles de activación de componentes del sistema inmunitario innato y adaptativo con un estado de inflamación crónica sistémico, característico de los pacientes bajo un TARc supresor, que explicaría el desarrollo y/o debut clínico de las ECAs en estos pacientes (Figura 2). Futuros estudios en grandes cohortes ayudarán a esclarecer si los factores inmunológicos observados en estos trabajos podrían mejorar los actuales algoritmos predictores de riesgo cardiovascular, como el índice de Framingham, cuya capacidad discriminatoria de estas enfermedades continua siendo objeto de debate (54, 55).

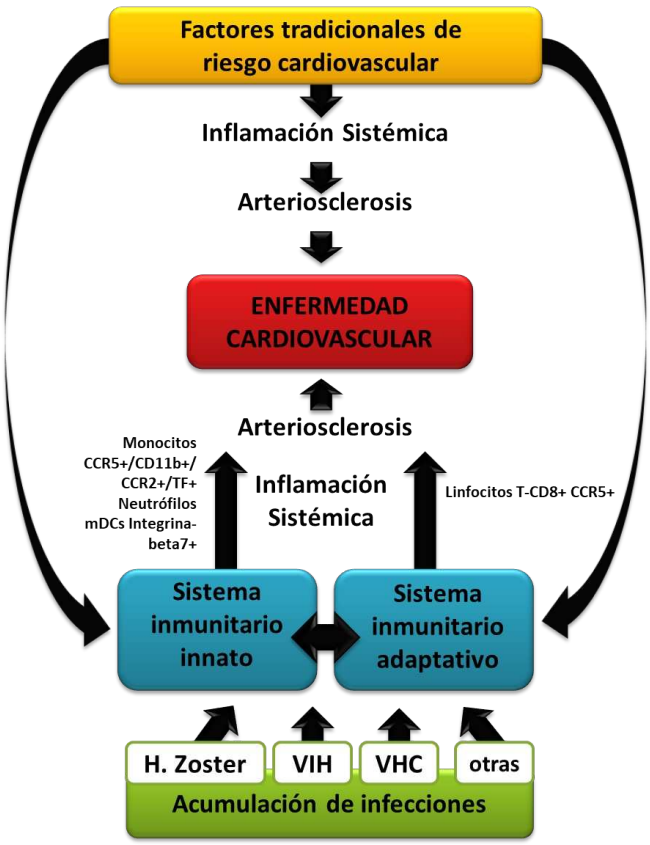

Figura 2. Diagrama esquemático de los factores implicados en el desarrollo de enfermedades cardiovasculares.

Una cuestión destacable derivada de estos resultados es que nos han permitido confirmar evidencias previas sobre la implicación del receptor CCR5 y sus ligandos en la progresión de distintas ECAs (56–58). Estos datos refuerzan la importancia de este receptor en el desarrollo de estrategias terapéuticas (59, 60). Agonistas de CCR5, como Maraviroc, además de controlar la infección impidiendo la entrada del virus en la célula, tienen relevantes efectos inmunomoduladores (61–63) y podrían revertir el perfil proinflamatorio que predomina en el desarrollo de ECAs, interfiriendo con la activación y/o migración de monocitos y linfocitos T con altos niveles de expresión de CCR5. Además, el bloqueo del receptor CCR5 o modificaciones en la expresión, podría disminuir la replicación viral y el reservorio del VIH, preservando de esta manera el sistema inmunológico del paciente, a pesar de que existen controversias en cuanto a este respecto (64, 65). Esta idea explicaría, en parte, la menor progresión clínica que hemos observado en los pacientes portadores de la delecion CCR5Δ32 en heterocigosis. Actualmente, ambiciosos proyectos como el del consorcio IciStem estudian el posible mecanismo de erradicación del VIH tras trasplantes de células madre a partir de donantes portadores de la deleción CCR5Δ32 basándose en el único caso conocido de curación del VIH, el caso de “El Paciente Berlín”(66).

Todo lo expuesto hasta ahora se ha centrado en lo que ocurre en la mayoría de pacientes infectados por VIH. Curiosamente, estos factores inmunológicos implicados en el desarrollo de ECAs también se observan en los mecanismos responsables del control espontáneo en pacientes controladores del VIH.

Distintas observaciones que han descrito una progresión de la enfermedad en los pacientes controladores, como desarrollo de morbilidades, inflamación, así como una proporción variable de

pacientes que pierden el control virológico en el tiempo (49, 50), han hecho que los controladores hayan pasado de ser un grupo homogéneo de pacientes capaces de controlar espontáneamente el VIH durante largo tiempo sin TARc (38), a considerarse un grupo con una gran heterogeneidad clínica. Todos estos estudios ponen en peligro la opinión general de la comunidad científica de que los controladores de élite podrían representar un modelo natural para el desarrollo de la cura funcional del VIH (67). Por ello, una de las principales líneas de trabajo de nuestro grupo de investigación es identificar fenotipos extremos dentro de los pacientes controladores que nos permitan delimitar el grupo de sujetos que mejor se ajuste al modelo de cura funcional.

Una de las causas de progresión de la enfermedad es la pérdida progresiva de linfocitos T-CD4+ que puede llegar a ser motivo de inicio de un TARc en los pacientes controladores. Recientemente hemos identificado una asociación genotípica entre el genotipo HLA-B57 y polimorfismos asociados a IFNL4 y un fenotipo extremo de controladores, los "Long Term Non-Progressors" (LTNP), el cual describe aquellos controladores que conservan niveles sostenidos de linfocitos T CD4 por encima de 500 células/ mm^3 durante al menos 7 años desde el diagnóstico (42). La otra causa de progresión en un paciente controlador que también determina el inicio de un TARc es la pérdida de control virológico. La presente Tesis Doctoral se ha centrado en analizar los mecanismos implicados en esta progresión virológica llevando a cabo un exhaustivo estudio longitudinal analizando factores inmunológicos y virológicos que se asociaban con la pérdida de control virológico en los CE. Desde el punto de vista inmunológico, los controladores transitorios (CT) que perdían el control, presentaron una menor respuesta T-VIH-específica así como por una menor polifuncionalidad (producción simultánea de múltiples citoquinas en respuesta

al VIH) en comparación con los controladores persistentes (CP), los cuales mantenían niveles altos de respuesta a lo largo del seguimiento. Mediante secuenciación de las secuencias víricas *gag* y *env*, observamos que los CP presentaban muy baja diversidad y evolución en sus secuencias en el tiempo. La ausencia de replicación desde la infección aguda podría sugerir una remisión virológica persistente e incluso una hipótesis más provocativa aún es que estos individuos podrían haber conseguido la erradicación del virus de manera espontánea. El conocimiento de los mecanismos implicados en este control persistente podrá ayudar al diseño de estrategias terapéuticas en los próximos años.

Atendiendo a estos datos, hemos propuesto como modelo de cura funcional al paciente controlador de élite LTNP y que no pierde el control virológico: “Long-Term Persistent Elite Controllers” (41) (Figura 3).

Una correcta caracterización de este “Controlador de élite persistente” también es esencial para la práctica clínica. No está claro en las actuales guías de tratamiento si los CE deben ser tratados con TARc (68, 69). Es plausible pensar que controladores transitorios, que pierden su capacidad de control a corto plazo, podrían beneficiarse del TARc gracias a la identificación de biomarcadores predictivos. Para abordar esta hipótesis, en la presente Tesis Doctoral determinamos un perfil de citoquinas y proteínas plasmáticas proinflamatorias, así como un perfil metabolómico caracterizado por una disfunción mitocondrial, asociados a la pérdida de control virológico. Curiosamente, la citoquina proinflamatoria RANTES fue uno de los principales biomarcadores predictores de pérdida de control, con una gran capacidad de discriminación entre CT y CP. Los altos niveles de este ligando natural de CCR5 ampliamente estudiado en la infección por VIH (70, 71), podrían reflejar bajos niveles residuales de

replicación viral en los CT que se asociaría con la disfunción también observada en las células T (72). Este hallazgo no sólo define RANTES como biomarcador predictivo de pérdida de control virológico sino que, una vez más, sugiere la inmunomodulación de éste y su receptor CCR5 como diana terapéutica también en este escenario en la infección.

Con los trabajos aquí presentados hemos sido pioneros en el empleo de las emergentes técnicas "ómicas" en el escenario de los CE. Las asociaciones encontradas entre los niveles de proteínas proinflamatorias y metabolitos plasmáticos con la respuesta inmunitaria refuerzan la importancia el reciente campo de estudio denominado "inmunometabolismo", el cual propone el estudio de las vías metabólicas intrínsecamente asociadas al estado del sistema inmunitario. Además, estas aproximaciones -ómicas, gracias a la gran capacidad discriminatoria que establecen entre CP y CT, nos han permitido salvar una de las principales limitaciones de este estudio: el bajo recuento muestral. La baja representación de estos grupos de pacientes y el diseño longitudinal retrospectivo del estudio hacen que estos trabajos sean únicos a nivel internacional. Esta tesis no hubiera sido posible sin el trabajo colaborativo de los distintos hospitales y grupos de investigación que formamos parte del Grupo de Estudio de Pacientes Controladores de la Red Española de Investigación en SIDA (EC-RIS) junto con el soporte del Biobanco nacional de muestras VIH (BB-RIS).

En resumen, en la presente Tesis Doctoral hemos identificado factores inmunológicos asociados a la inmunopatogenia y al control de la infección del VIH. Estos factores se han asociado al desarrollo de ENOS cardiovasculares en pacientes VIH bajo TARc supresor y a la progresión virológica en pacientes controladores de élite. Los datos derivados de estos estudios podrán servir como base en el

diseño de estrategias inmunoterapéuticas en la práctica clínica de estos pacientes con objeto de evitar el desarrollo de ENOS y, para conseguir la remisión virológica persistente o erradicación del VIH tomando como modelo el controlador de élite persistente.

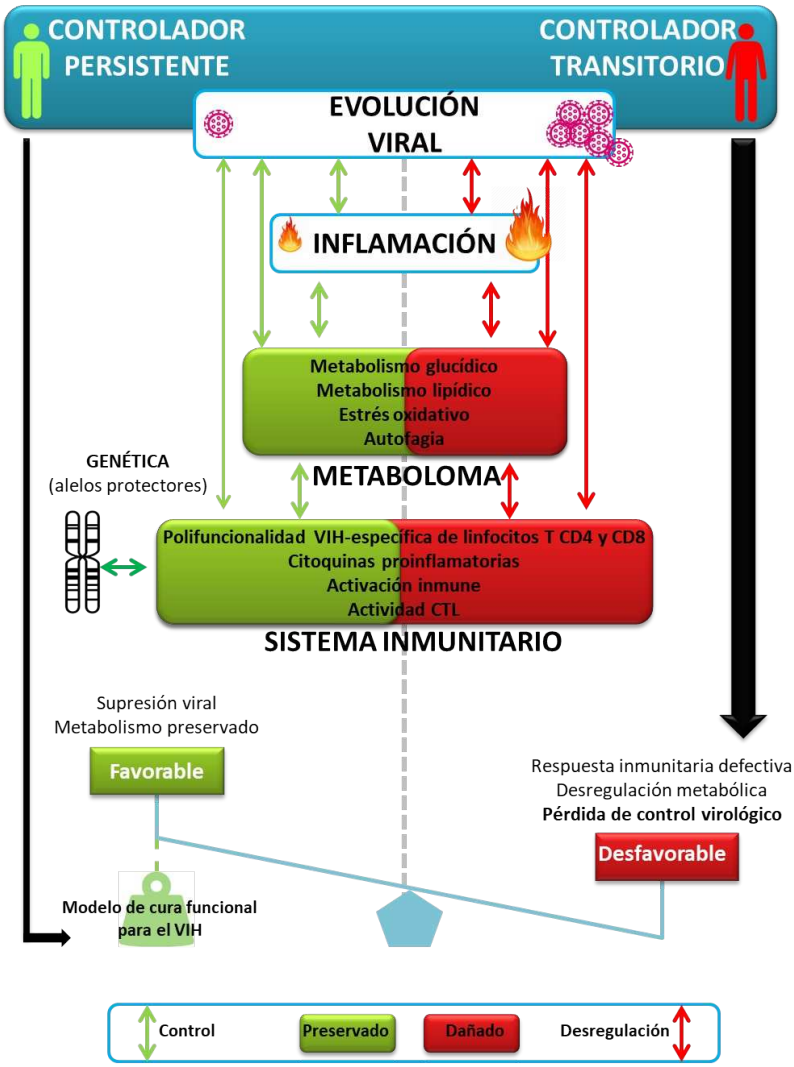

Figure 3. Relación entre el inmunometabolismo e inflamación en controladores de élite persistentes y transitorios. Los controladores persistentes representan el mejor modelo para el desarrollo de vacunas y estrategias inmunoterapéuticas destinadas a conseguir la cura funcional de la infección por el VIH.

CONCLUSIONES

1. La activación de linfocitos T-CD8+ y monocitos precede la aparición de síndrome coronario agudo en pacientes infectados por VIH. En este escenario, la asociación entre el sistema inmunitario adaptativo e innato refleja una progresiva desregulación homeostática que implica la activación de estos tipos celulares, como ocurre con el aumento de expresión de marcadores de superficie como CCR5 susceptibles de ser dianas terapéuticas en la infección.

2. El polimorfismo Asp299Gly TLR4 asociado al desarrollo de ECAs podría explicar el perfil proinflamatorio y la activación observada en los monocitos implicados en el desarrollo de estas patologías.

3. Tanto la coinfección por VHC como el efecto acumulativo de distintas infecciones víricas y bacterianas juegan también un papel importante en el desarrollo de ECAs en pacientes infectados por el VIH.

4. La deleción en heterocigosis CCR5Δ32 es un factor protector asociado a la supervivencia a largo plazo, en sujetos bajo TARc. Estos datos refuerzan de nuevo la importancia del receptor CCR5 como diana terapéutica para evitar la progresión de la enfermedad en pacientes bajo TARc.

5. El análisis de los factores implicados en el control persistente del VIH, caracterizados por bajos niveles de marcadores inflamatorios, baja diversidad viral y preservada respuesta inmunitaria, nos han permitido delimitar un nuevo fenotipo extremo de controlador que podrá ser utilizado como modelo de cura funcional.

6. Diferencias en los niveles de proteínas plasmáticas y alteraciones en las vías de señalización en las que se encuentran implicadas determinan un estado inflamatorio que precede la pérdida de control espontáneo del VIH. Estas proteínas pueden ser consideradas como potenciales biomarcadores predictivos de la pérdida de control.

7. Un perfil metabolómico específico, caracterizado por un mayor estrés oxidativo y disfunción mitocondrial, precede la pérdida de control virológico en CE. Existen fuertes asociaciones entre estos metabolitos y lípidos específicos con parámetros de la respuesta inmunológica defectiva característica también de estos pacientes.

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ANEXOS

Otros trabajos publicados durante el periodo de Tesis Doctoral:

1. Dominguez-Molina B, Ferrando-Martinez S, **Tarancon-Diez L**, Hernandez-Quero J, Genebat M, Vidal F, Muñoz-Fernandez MA, Leal M, Koup R, Ruiz-Mateos E. Immune correlates of natural HIV elite control and simultaneous HCV clearance-supercontrollers. Front Immunol. 2018. In press.

2. Dominguez-Molina B, Machmach K, Perales C, **Tarancon-Diez L**, Gallego I, Shedon J, Leal M, Domingo E, Ruiz-Mateos E. 2018. Toll Like Receptor-7 and -9 agonists improve Hepatitis C Virus replication and infectivity inhibition by plasmacytoid dendritic cells. J Virol. 2018. In press.

3. Herrero-Fernandez I, Rosado-Sanchez I, Genebat M, **Tarancon-Diez L**, Rodriguez-Mendez MM, Pozo-Balado MM, Lozano C, Ruiz-Mateos E, Leal M, Pacheco YM. 2018. Improved CD4 T cell profile in HIV-infected subjects on maraviroc-containing therapy is associated with better responsiveness to HBV vaccination. J Transl Med 16:238.

4. Vitalle J, Terren I, Gamboa-Urquijo L, Orrantia A, **Tarancon-Diez L**, Genebat M, Ruiz-Mateos E, Leal M, Garcia-Obregon S, Zenarruzabeitia O, Borrego F. 2018. Altered Expression of CD300a Inhibitory Receptor on CD4+ T Cells From Human Immunodeficiency Virus-1-Infected Patients: Association With Disease Progression Markers. Front Immunol 9:1709.

5. **Tarancon-Diez L**, Dominguez-Molina B, Viciana P, Lopez-Cortes L, Ruiz-Mateos E. 2018. Long-term Persistent Elite HIV-controllers: The Right Model of Functional Cure. EBioMedicine 28:15–16.

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8. Rosado-Sanchez I, Herrero-Fernandez I, **Tarancon-Diez L**, Moreno S, Iribarren JA, Dalmau D, Vera-Mendez F, Leal M, Pacheco YM. 2018. Increased frequencies of Th17 cells and IL17a-producing regulatory T-cells preceding the immunodiscordant response to antiretroviral treatment. J Infect 76:86–92.

9. Dominguez-Molina B, **Tarancon-Diez L**, Leal M, Ruiz-Mateos E. 2017. Reply to Kuniholm et al. Clin Infect Dis 65:1244–1245.

10. Dominguez-Molina B, **Tarancon-Diez L**, Hua S, Abad-Molina C, Rodriguez-Gallego E, Machmach K, Vidal F, Tural C, Moreno S, Goni JM, Ramirez de Arellano E, Del Val M, Gonzalez-Escribano MF, Del Romero J, Rodriguez C, Capa L, Viciana P, Alcami J, Yu XG, Walker BD, M L, Lichterfeld M, Ruiz-Mateos E. 2017. HLA-B*57 and IFNL4-related polymorphisms are associated with protection against HIV-1 disease progression in controllers. Clin Infect Dis 64:621–628.

11. Gonzalez-Serna A, Genebat M, De Luna-Romero M, **Tarancon-Diez L**, Dominguez-Molina B, Pacheco YM, Munoz-Fernandez MA, Leal M, Ruiz-Mateos E. 2016. Validation of the HIV Tropism Test TROCAI Using the Virological Response to a Short-Term Maraviroc Monotherapy Exposure. Antimicrob Agents Chemother 60:6398–6401.

Propiedad intelectual desarrollada durante la tesis:

Title registered industrial property: Methods for the prognosis of HIV-infected subjects

Inventors/authors/obtainers: Francesc Vidal Marsal; Ezequiel Ruiz-Mateos; Joaquim Peraire Forner; Consuelo Viladés Laborda; Anna Rull Aixa; Esther Rodríguez Gallego; Luis Fernando López-Cortés; Verónica Alba Elvira; Laura Tarancón Díez

Entity holder of rights: Institut d'Investigació Sanitària Pere Virgil, Universitat Rovira i Virgili, Servicio Andaluz de Salud

Nº of application: P16131EP00

Country of inscription: Spain

Date of register: 18/09/2018

Otros trabajos publicados:

1. **Tarancon Diez L**, Bonsch C, Malkusch S, Truan Z, Munteanu M, Heilemann M, Hartley O, Endesfelder U, Furstenberg A. 2014. Coordinate-based co-localization-mediated analysis of arrestin clustering upon stimulation of the C-C chemokine receptor 5 with RANTES/CCL5 analogues. Histochem Cell Biol 142:69–77.

2. Truan Z, **Tarancon-Diez L,** Bonsch C, Malkusch S, Endesfelder U, Munteanu M, Hartley O, Heilemann M, Furstenberg A. 2013. Quantitative mophological analysis of arrestin2 clustering upon G protein-coupled receptor stimulation by super-resolution microscopy. Journal of structural biology 184:329-334.

DICTAMEN ÚNICO EN LA COMUNIDAD AUTÓNOMA DE ANDALUCÍA

D/Dª: Jose Salas Turrents como secretario/a del CEI de los hospitales universitarios Vírgen Macarena-Virgen del Rocío

CERTIFICA

Que este Comité ha evaluado la propuesta de (No hay promotor/a asociado/a) para realizar el estudio de investigación titulado:

TÍTULO DEL ESTUDIO:	Papel de las células plasmacitoides dendríticas en el control espontáneo de la replicación del VIH y en su patogénesis. Relevancia clínica de los mecanismos implicados ,(pDC en el control y patogénesis del VIH)
Protocolo, Versión:	22/04/2016
HIP, Versión:	22/04/2016
CI, Versión:	22/04/2016

Y que considera que:

Se cumplen los requisitos necesarios de idoneidad del protocolo en relación con los objetivos del estudio y se ajusta a los principios éticos aplicables a este tipo de estudios.

La capacidad del/de la investigador/a y los medios disponibles son apropiados para llevar a cabo el estudio.

Están justificados los riesgos y molestias previsibles para los participantes.

Que los aspectos económicos involucrados en el proyecto, no interfieren con respecto a los postulados éticos.

Y que este Comité considera, que dicho estudio puede ser realizado en los Centros de la Comunidad Autónoma de Andalucía que se relacionan, para lo cual corresponde a la Dirección del Centro correspondiente determinar si la capacidad y los medios disponibles son apropiados para llevar a cabo el estudio.

Lo que firmo en SEVILLA a 21/12/2016

D/Dª. Jose Salas Turrents, como Secretario/a del CEI de los hospitales universitarios Vírgen Macarena-Virgen del Rocío

CERTIFICA

Que este Comité ha ponderado y evaluado en sesión celebrada el 24/11/2016 y recogida en acta 13/2016 la propuesta del/de la Promotor/a (No hay promotor/a asociado/a), para realizar el estudio de investigación titulado:

TÍTULO DEL ESTUDIO:	Papel de las células plasmacitoides dendríticas en el control espontáneo de la replicación del VIH y en su patogénesis. Relevancia clínica de los mecanismos implicados ,(pDC en el control y patogénesis del VIH)
Protocolo, Versión:	22/04/2016
HIP, Versión:	22/04/2016
CI, Versión:	22/04/2016

Que a dicha sesión asistieron los siguientes integrantes del Comité:

Presidente/a
D/Dª. Víctor Sánchez Margalet

Vicepresidente/a
D/Dª. Dolores Jiménez Hernández

Secretario/a
D/Dª. Jose Salas Turrents

Vocales
D/Dª. Enrique Calderón Sandubete
D/Dª. Francisco Javier Bautista Paloma
D/Dª. Gabriel Ramírez Soto
D/Dª. Carlos García Pérez
D/Dª. Juan Ramón Lacalle Remigio
D/Dª. Joaquin Quiralte Enriquez
D/Dª. Cristina Pichardo Guerrero
D/Dª. Javier Vitorica Fernandez
D/Dª. Juan Carlos Gomez Rosado
D/Dª. Luis Lopez Rodriguez
D/Dª. Enrique de Álava Casado
D/Dª. EVA MARIA DELGADO CUESTA
D/Dª. ANGELA CEJUDO LOPEZ
D/Dª. M LORENA LOPEZ CERERO
D/Dª. Amancio Carnero Moya
D/Dª. Manuel Ortega Calvo
D/Dª. LUIS GABRIEL LUQUE ROMERO
D/Dª. ANTONIO PÉREZ PÉREZ
D/Dª. María Pilar Guadix

Que dicho Comité, está constituido y actua de acuerdo con la normativa vigente y las directrices de la Conferencia Internacional de Buena Práctica Clínica.

Lo que firmo en SEVILLA a 21/12/2016

Código Seguro De Verificación:	d41e7c4d04a90e05d16e1c965b916d1525e30ee9	Fecha	21/12/2016
Normativa	Este documento incorpora firma electrónica reconocida de acuerdo a la Ley 59/2003, de 19 de diciembre, de firma electrónica.		
Firmado Por	Jose Salas Turrents		
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Servicio Andaluz de Salud
CONSEJERÍA DE IGUALDAD, SALUD Y POLÍTICAS SOCIALES

Hospital Universitario VIRGEN DEL ROCIO.
Avda. Manuel Siurot s/n 41013 SEVILLA

INFORME DEL COMITÉ DE ÉTICA DE LA INVESTIGACIÓN DE CENTRO H.U. VIRGEN DEL ROCIO

El Comité de Ética de la Investigación de Centro H.U. Virgen del Rocío de Sevilla, en Sesión celebrada el día dieciocho de diciembre de dos mil doce (Acta 11/13):

1. Ha procedido a la revisión del estudio:

   - Código de CEI **2013PI/356**
   - Presentado por **D. Manuel Leal Noval**
   - Titulado: "**Análisis de la cinética y función de las células plasmacitoides dendríticas y la respuesta celular T en el control espontáneo del VIH-1**"

2. Tras su valoración procede a emitir **INFORME FAVORABLE** del mismo.

Y para que conste y surta los efectos oportunos se expide la presente certificación en Sevilla, a 16 de enero de dos mil catorce

CONSEJERÍA DE SALUD
Servicio Andaluz de Salud
Comité de Ética de la Investigación de Centro
H.U. Virgen del Rocío
SEVILLA

Fdo.: Fco. Javier Bautista Paloma
Presidente del CEI

INFORME DEL COMITÉ DE ÉTICA DE LA INVESTIGACIÓN DE CENTRO H.U. VIRGEN DEL ROCIO

El Comité de Ética de la Investigación de Centro H.U. Virgen del Rocío de Sevilla, en Sesión celebrada el día veintiocho de noviembre de dos mil doce (Acta 10/12):

1. Ha procedido a la revisión del estudio:

   - Código de CEI **2012PI/240**
   - Presentado por **D. Ezequiel Ruiz-Mateos Carmona**
   - Titulado **"Papel de las células plasmacitoides dendríticas y las respuesta celular T en el control espontáneo del VIH-1 y del virus de la hepatitis C en pacientes co-infectados"**

2. Tras su valoración procede a emitir **INFORME FAVORABLE** del mismo.

Cód. 010470

Y para que conste y surta los efectos oportunos se expide la presente certificación en Sevilla, a diez de diciembre de dos mil doce

Fdo.: Fco. Javier Bautista Paloma
Presidente